Antiviral Research, 23 (1994) 33–44

SSDI 0166-3542(94)E0056-Y

AVR 00670

Antiviral Research

A slow release formulation for recombinant bovine interferon α_I-1

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(Received 28 December 1992; accepted 20 September 1993)

Summary

Recombinant bovine interferon-α_I1 (rBoIFN-α) has known antiviral and immunomodulatory effects which have been exploited to reduce clinical disease in a number of clinical situations including bovine respiratory diseases. A slow release rBoIFN-α formulation may be of value to reduce bovine respiratory disease under field conditions by extending the period of protection, and hence improving the prophylactic benefits of rBoIFN-α. In this report, we describe a formulation of rBoIFN-α in sesame oil containing calcium stearate which can successfully sustain the release of rBoIFN-α over an 8-day period. Recombinant bovine IFN-α could be measured in serum for 8 days following treatment with an initial burst of release 6 h after injection. After a single subcutaneous depot injection of 50 mg and 100 mg of rBoIFN-α, initial serum levels reached 12–15 ng/ml and 25 ng/ml respectively. Correlating with this burst of release, there was a decrease in the number of circulating $CD4^-CD8^-$ $\gamma\delta^+$ T lymphocytes, and a slight neutropenia. No alterations in other cell phenotypes tested (CD4, CD8, CD2, CD6, B cells, monocytes or MHC class II) were observed, nor were there changes in lymphokine activated killer (LAK), natural killer (NK) cell activity, or oxygen radical formation (assessed by reduction of nitroblue tetrazolium). However, despite the rapid and short-lived burst of rBoIFN-α, levels of 2–5 oligoadenylate (2–5 A) synthetase remained elevated for 8 days. The sustained increase of 2–5 A synthetase was not due to the high initial dose released during the burst 6–12 h after injection, since injection of a bioavailable equivalent dose of interferon induced a significant rise in 2–5 A synthetase activity for 4 days only. As 2–5 A synthetase is known to be a correlate of antiviral activity, we propose that this formulation of

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rBoIFN-α may be one approach to increase the window of protection, leading to more effective prevention of bovine respiratory disease.

Bovine α-interferon; α-Interferon; Release rate

Introduction

Interferons have been classified as α, β or γ, on the basis of cell origin, antigenic specificities and more recently, gene organization. The gene encoding bovine IFN-α_I1 has been cloned and expressed at high levels and has been assessed a variety of biological activities (Capon et al, 1985; Czarniecki et al., 1986). The antiviral state that recombinant bovine (rBo) IFN-α can induce in vitro has been demonstrated to be greater than that induced by rBoIFN-γ (Czarniecki et al., 1986). This and the fact that purified rBoIFN-α is available in large quantities has made this cytokine an excellent candidate as a veterinary antiviral agent.

A number of studies have demonstrated that rBoIFN-α can protect cattle against shipping fever using a bovine herpesvirus type 1 (BHV-1)-*Pasteurella haemolytica* model. A significant protective effect was noticed when animals were treated with rBoIFN-α and then infected under controlled conditions, although when the same treatment was assessed under field conditions, there was a diminished effect (Babiuk et al., 1987). This appeared to be due to the short-lived effect of rBoIFN-α (i.e., less than 7 days) and to the fact that the window of susceptibility under field conditions exceeded the protective effects of rBoIFN-α. It appears that more effective prevention of shipping fever by immunomodulation using rBoIFN-α could be successfully achieved with a slow release formulation (Babiuk et al., 1991).

A formulation in an oily vehicle appears to be a valuable approach toward sustaining the release of cytokines, including rBoIFN-α. While we have not investigated in vivo protection against challenge, we did observe a sustained elevation of 2–5 oligoadenylate (2–5 A) synthetase activity, an enzymatic marker which is known to correlate with antiviral activity (McMahon and Kerr, 1983; Bielefeldt-Ohmann et al., 1989).

Materials and Methods

Animals

Initial studies on the pharmacokinetics of rBoIFN-α slow release were carried out using 15 outbred calves of approximately 12–18 months of age. In other studies, two groups of genetically identical Holstein calves obtained by embryo splitting were used; their ages were 14 months (3 animals) and 6–8 months (4 animals).

Interferon-α

Recombinant bovine interferon-α_I1 (rBoIFN-α) was formulated in sesame oil containing lyophilized protein and calcium stearate. Doses of 100 mg or 50 mg in volumes of 4 ml or 2 ml, respectively, were administered subcutaneously. The bioavailability of rBoIFN-α in this suhcutaneous formulation was estimated at approximately 33% (Rossow et al., unpublished data). Therefore, an intramuscular bolus injection of 17 mg rBoIFN-α in PBS was used to compare the in vivo effects of both formulation types.

Serological assays for rBoIFN-α

Radioimmunoassay. Radioiodination of interferon was carried out using iodogen. Briefly, 95 μl of 0.5 M sodium phosphate buffer (pH 7.4) and 3 μl $Na^{125}I$ (approx. 1 mCi) were added to a tube containing 7.5 μg iodogen. After 15 s, 7 μg rBoIFN-α was placed in the tube and after mixing, the reaction was allowed to proceed for 20 min. The product was separated on a Polyprep *G* column equilibrated with elution buffer [0.05 M phosphate-buffered saline (PBS) containing 0.2% (w/v) bovine serum albumin (BSA)]. The eluted fractions containing the initial peak of activity were pooled and applied onto a Sephadex G-100 column equilibrated with PBS containing 0.2% BSA. The eluate fractions containing the major peak of radioactivity were pooled and used in the radioimmunoassay. The specific activity of the labelled preparations averaged 130 μCi/μg of BoIFN-α. The unlabelled interferon standard prepared in bovine serum was from the same lot of rBoIFN-α. Standard and serum samples (100 μl) were pipetted in duplicate into the assay tubes, followed by the additions of the 250 μl of a rabbit anti-rBoIFN-α (1:500 000) antibody and radiolabelled interferon (100 μl; approx. 10 000 cpm or approx. 13 000 dpm). Tubes were vortexed and incubated overnight at 4°C. A second precipitating antibody was then added undiluted as supplied by the manufacturer. After 1 h incubation at 4°C, the tubes were centrifuged for 20 min at $10\,000 \times g$, the supernatants discarded, and the radioactivity contained in the precipitates determined by γ-spectrometry. The limit of detection of the assay ranged from 0.05 to 0.1 ng of interferon per tube; inter- and intra-assay coefficients of variation of the assay were less than 15% and 10%, respectively.

rBoIFN-α capture ELISA. Two monoclonal antibodies against rBoIFN-α were used to coat Immulon II ELISA plates at a 1:1000 dilution in sodium carbonate buffer at pH 9.6. Following washing in PBS with 0.05% Tween 20 (PBST), wells were blocked with PBST containing 0.1% gelatin and washed six times. Serum samples (200 μl) were added, and rBoIFN-α was used as a control in the concentration range of 1000 pg to 10 pg/ml. Plates were incubated at 37°C for 2 h. Following 6 washes in PBST, rabbit anti-rBoIFN-α (lot 2656-22a, CIBA-GEIGY) was added at 1:4000 and incubated for 45 min at 37°C to detect bound rBoIFN-α. Biotinylated anti-rabbit serum was then added, followed by horseradish peroxidase conjugated streptavidin-biotinylated complex

(Amersham, Mississauga, ON). Diluent and wash solution was PBST. Substrate for the enzyme reaction was 15 μg/ml ABTS in sodium citrate buffer, pH 4.0. Absorbance was read at 405 nm using a BioRad Model 3550 microplate reader.

Cytotoxic assays. Lymphokine activated killer (LAK) cell activity was assessed in an 18 h ^{51}Cr release assay in which K562 target cells were labelled with $Na_2{}^{51}CrO_4$ (Amersham Canada Ltd., Oakville, ON), and cultured in the presence or absence of 100 U/ml human recombinant IL-2 (Boehringer-Mannheim) in minimal essential medium (MEM) (Campos and Rossi, 1986). Effector (PBMC) cells, isolated from whole blood, were added to cultures at an effector to target (E:T) ratio of 100:1. Control cultures comprised target cells cultured in the presence of medium alone (spontaneous release) or Triton X-100 (maximum release). Supernatants were harvested using an appropriate harvesting system (Skatron, Sterling, VA, USA). The percentage specific cytotoxicity was calculated from the mean of quadruplicate cultures using the following formula:

$$\% \text{ cytotoxicity} = \frac{\text{cpm with effector cells} - \text{cpm spontaneous release cpm total release}}{\text{cpm spontaneous release}} \times 100$$

Production of peripheral blood mononuclear cell extracts. PBMC were isolated from buffy coats by Ficoll-Hypaque density gradient centrifugation as previously described (Hughes et al., 1988). 10^7 cells were pelleted in 1.8 ml Eppendorf tubes at $11\,000 \times g$ for 5 s and all fluid was removed. Following resuspension in lysing buffer B (Gresser et al., 1985), cells were incubated on ice for 30–60 min, centrifuged at $11\,000 \times g$ for 5 min, and stored at −70°C until the assays were performed.

Determination of 2–5 A synthetase activity in PBMC. The method described by Bielefeldt-Ohmann et al. (1989) was used for the determination of 2–5 A synthetase. Briefly, aliquots of 10 μl washed type 6 poly(rI):(rC)-agarose beads (Pharmacia) were mixed with 10 μl of cytosol or buffer [Buffer C (Gresser et al., 1985)] and incubated at 30°C for 15 min. After washing in buffer, 10 μl of substrate (3 mM ATP, 1.6 μCi [α-^{32}P]ATP (Amersham, 410 Ci/mmol), 10 mM creatine phosphate, creatine kinase (3 mg/ml), and poly (rI):(rC) (40 μg/ml) in buffer) were added. Samples were incubated for 20 h at 30°C. The reaction was terminated by the addition of 1 unit of bacterial alkaline phosphatase (Sigma) in 40 μl of 0.1 M Tris (pH 10). Following incubation for 2 h at 37°C, the beads were pelleted and 20 μl of supernatant was applied to a 0.3 ml column of acid alumina (type WA-1). The oligoadenylate phosphate-resistant cores were eluted with 3 ml of 1 mM glycine-HCl (pH 2) and counted by Cherenkov radiation in a scintillation counter (Beckman, Richmond, B.C.).

Flow cytometry

Following isolation from blood, PBMC were stained for flow cytometric analysis using monoclonal antibodies (Veterinary Medical Research and Development Inc., Pullman, WA, USA) against the following phenotypic markers: CD2, CD4, CD6, CD8, $\gamma\delta^+$ T cells (B7A, BAQ4A and CACTB6A), B cells (BAQ44A), BoLA Class II (non-polymorphic determinant, TH14B), and monocytes (DH59B) (Davis et al., 1987). The leukocyte populations were stained with the lineage-specific monoclonal antibodies and FITC goat anti-mouse IgG (Becton Dickinson, Mountain View, CA, USA) as previously described (Griebel et al., 1988). Cells were analysed with an EPICS C Flow Cytometer (Coulter Electronics Ltd., Hialeah, FL, USA). Data from 10 000 cells was collected, and two-parameter analyses of forward angle light scatter versus 90° light scatter was used to gate the population for fluorescence analysis. Using cells stained with a second antibody only, a threshold fluorescent intensity was determined, above which cells were considered positive.

Clinical evaluation

Animals were observed for 8–9 days following treatment. Complete blood counts and differentials were done and rectal temperatures were measured on a daily basis throughout the experiment.

Results

Clinical effects of rBoIFN-α

Apart from pyrexia observed when the 100 mg formulation was assessed, the slow release formulations used in this study did not induce any overt signs typical of high dose rBoIFN-α therapy. When a dose of 50 mg was injected, no pyrexia, diarrhea or depression was observed. Hematologically, all animals that received rBoIFN-α showed a slight neutropenia; other white cell counts dropped slightly, but remained within normal limits. Following injection of 17 mg rBoIFN-α i.m. as a bolus, pyrexia and a transient neutropenia occurred at 6 h. However, all white cell counts returned to normal ranges 24 h after injection. None of the animals given the 17 mg bolus injection showed any other clinical sign.

Flow cytometry

Following administration of 50 mg rBoIFN-α in the slow release formulation or the 17 mg bolus injection, there was a consistent decrease in the number of $\gamma\delta^+$T cells as assessed by enumerating cells expressing either the WC1 or the WC2 phenotype (Fig. 1). This decrease in $\gamma\delta^+$ cells was maximal at 6–12 h after treatment. In all cases, the percentage of cells expressing $\gamma\delta^+$ T cell markers returned to normal 24 h after injection.

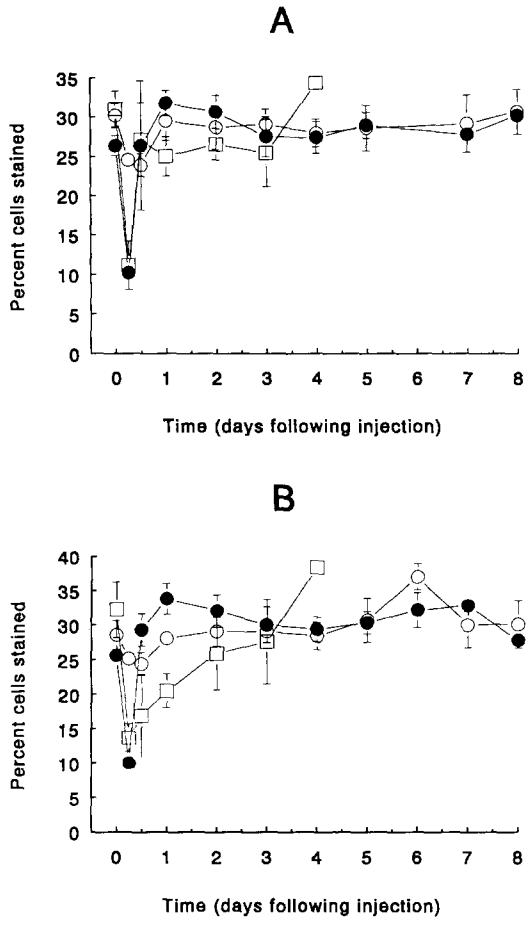


Fig. 1. Phenotypic profile of (A) $B7A^+$ ($WC1^+$) and (B) $CACTB6A^+$ ($WC2^+$) cells. Four genetically identical calves were treated with 50 mg rBoIFN-α in a slow release formulation at time 0. The number of cells stained per ml blood was calculated from the % cells stained (derived from 1 parameter gated histograms) and the number of leukocytes/ml (derived from complete blood counts). ●: slow-release rBoIFN-α-treated animals; ○: placebo (PBS) treated animals; □: 17 mg bolus rBoIFN-α-treated animals. Points represent the mean ± S.E.

Cytotoxicity

No significant changes were observed in the ability of PBMC to lyse labelled K562 targets following any of the rBoIFN-α treatments. Also, LAK activity assessed by adding IL-2 to cultures was not affected by rBoIFN-α treatment (data not shown).

Serum levels of rBoIFN-α

Initial experiments were carried out to determine the proper doses to use in the slow release formulation. Fifty- or 100-mg rBoIFN-α doses were injected

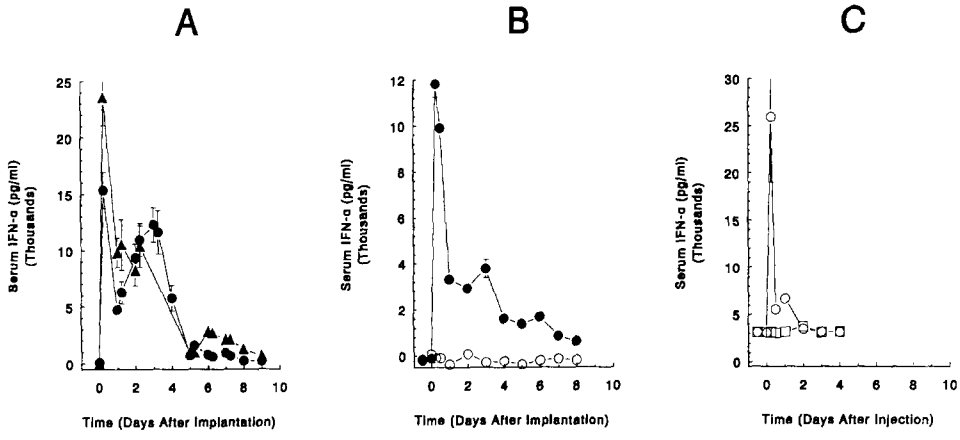
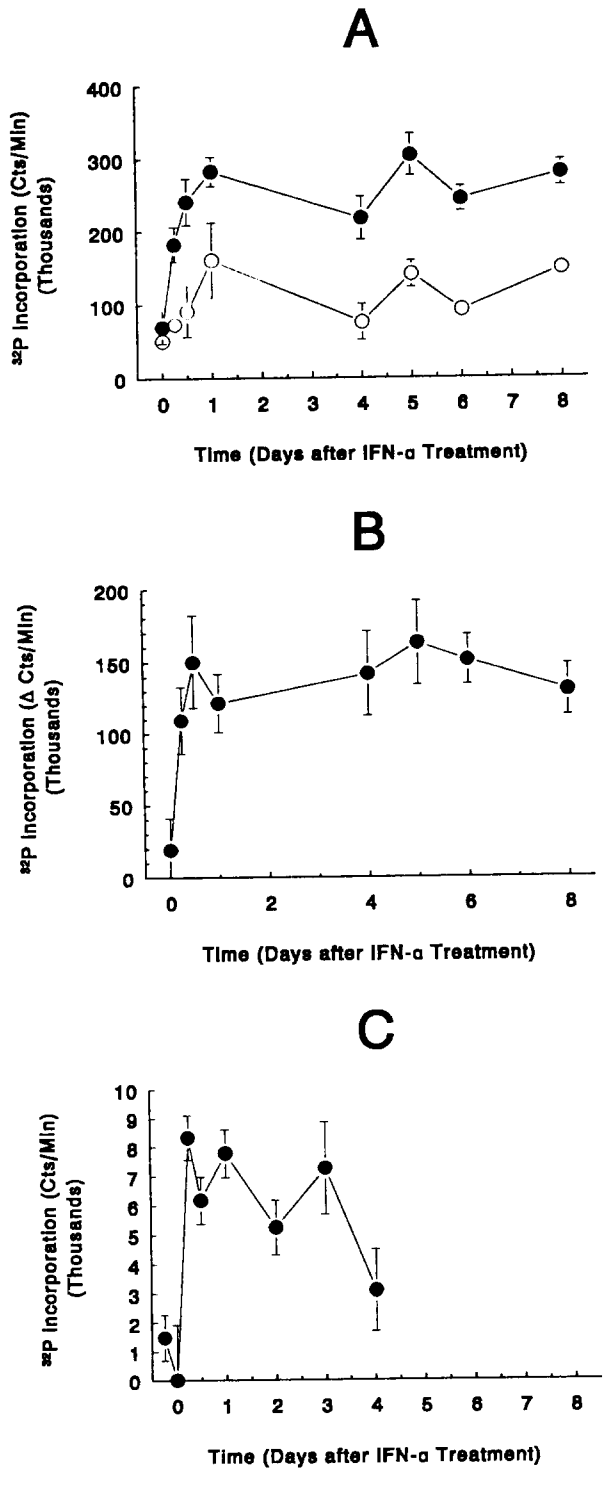


Fig. 2. Interferon levels detected by (A and B) radioimmunoassay (plasma) and (C) ELISA (serum). In A, animals were injected with either 50 mg (●) or 100 mg (▲), rBoIFN-α in a slow release formulation. In B, animals were injected with either 50 mg slow release IFN-α (●) or placebo (○). In C, animals were injected with a 17 mg bolus of IFN-α in PBS (□)or placebo (○). Points represent the mean ± S.E. of 5 to 10 outbred calves (A) or 3 genetically identical calves (B and C).

and plasma levels of rBoIFN-α were determined by RIA. In both instances, there was a distinct burst in plasma rBoIFN-α (Fig. 2A), which was of higher magnitude in those animals given 100 mg ($P<0.05$). However, in both cases, levels fell to below 5000 pg/ml by 5 days after injection, but stayed above serum IFN-α levels in control animals for the duration of the experiment. In a separate experiment, a rBoIFN-α capture ELISA was used and similar results were obtained following a 50-mg dose of rBoIFN-α. A burst occurred within 6 h of administration, followed by low but detectable levels for the remainder of this experiment (Fig. 2B). Since the duration of serum rBoIFN-α activity was prolonged for the same length of time regardless of dose, 50 mg was used for future experiments. In a third experiment, after injection of a 17-mg bolus of soluble rBoIFN-α (representing the bioavailable dose of the slow release formulation), there was a large initial peak of rBoIFN-α activity detectable in serum. This was followed by a rapid decline, with no detectable rBoIFN-α compared to controls after 48 h (Fig. 2C).

2–5 oligoadenylate synthetase activity

In all cases, 2–5 A synthetase activity increased dramatically following treatment, regardless of whether the rBoIFN-α was administered as a bolus or in a slow release form, reaching a maximum at 12 h post-treatment. In those animals receiving rBoIFN-α in the slow release formulation, levels of 2–5 A in PBMC were high 12 h following injection and remained high for the duration of the experiment (8 days) (Fig. 3A and B). In animals treated with the 17-mg bolus, levels of 2–5 A synthetase had fallen to normal levels (Fig. 3C) within 4


A
400
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32P Incorporation (Cts/Min)
(Thousands)
0 1 2 3 4 5 6 7 8
Time (Days after IFN-α Treatment)
B
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32P Incorporation (Δ Cts/Min)
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C
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32P Incorporation (Cts/Min)
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Time (Days after IFN-α Treatment)

days. In all cases, there were variations between sampling days, so that results are presented as increase above placebo-treated animals.

Discussion

Recombinant bovine rBoIFN-α has been shown to inhibit the in vitro replication of a number of viruses implicated in the bovine respiratory disease complex, and treatment of calves with this cytokine can increase their resistance to a combined viral/bacterial challenge consisting of BHV-1 and *P. haemolytica* (Babiuk et al., 1985; Czarniecki et al., 1985). However, a number of these experiments involved challenging calves within 2 or 3 days of treatment, and the beneficial effects of rBoIFN-α therapy are known to last for less than one week (Babiuk et al., 1991). These data explain the reduced efficacy of rBoIFN-α therapy under field conditions; animals do not all become infected at the same time relative to treatment, so the efficacy of therapy will be diminished. Therefore, the objective of this study was to examine an experimental sustained release formulation which would prolong the protective period of rBoIFN-α, in order to render timing of treatment less critical relative to disease development.

Interferon-α has multiple effects on lymphocyte trafficking and function. Alterations in the number of circulating T, B, and 'non-T-non-B' lymphocytes following IFN-α treatment have been reported (Griebel et al., 1989). Previous studies had indicated a drop in almost all the lymphocyte phenotypes tested following IFN treatment. In the present study, variations were observed in B macrophage and some T cell phenotypes subsequent to IFN-α slow release formulation administrations (data not shown). However, when these changes were compared to phenotypic changes among placebo-treated animals, there were no significant deviations in these values. Nevertheless, the number of circulating $B7A^+$ cells was significantly decreased as compared to controls. This change was of similar magnitude to that described previously (Griebel et al., 1989). We have found this to be the most significant phenotypic change demonstrated in peripheral blood following rBoIFN-α treatment. Originally, the B7A antibody was thought to detect a non-T-non-B lymphocytes phenotype (Griebel et al., 1989), but was subsequently established that it detects a molecule present on a subpopulation of mature $CD4^-$ $CD8^-$ TCR $\gamma\delta^+$ cells (WC1) (Morrison and Davis, 1991). Alterations in cells expressing

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Fig. 3. 2–5 A synthetase levels in PBMC following rBoIFN-α treatment. Cells were taken from placebo (○) or rBoIFN-α treated calves (□) and assessed for 2–5 A synthetase. (A) Following injection of 50 mg IFN-α in a slow release formulation, fluctuations occurred in both groups, though at all times, cells from rBoIFN-α-treated animals showed a significant increase. (B) When the results were expressed as change in 2–5 A synthetase compared to placebo treated animals, the trend was more apparent, with levels reaching a maximum after 12 h. (C) Following injection with 17 mg IFN-α, 2–5 A synthetase activity could be detected at significant levels for 4 days in PBMC. Results are expressed as mean increase above placebo-treated animals ± S.E.

this molecule and the $\gamma\delta$ TCR itself (CACTB6A, WC2) appear to be typical of rBoIFN-α therapy, as sometimes it appears to be the only phenotypic change in PBMC observed following rBoIFN-α therapy. Overall, there also appeared to be fewer cells expressing the WC2 phenotype, supporting the contention of a subset of WC1 cells (Morrison and Davis, 1991). As the number of $\gamma\delta^+$ cells is decreased by rBoIFN-α treatment, this may indicate that these cells may be sequestered in other sites or organs (e.g., mucosal surfaces and/or the skin). This fluctuation in peripheral $\gamma\delta^+$ appeared to occur, regardless of whether the cytokine was given in the slow release formulation or as a single injection (17 mg), implying that this immunoregulatory effect of rBoIFN-α is short-lived, and cannot be extended by changing the formulation. In other studies, we have shown that cells bearing this phenotype are uniquely sensitive to the effects of other cytokines such as IFN-γ and IL-2 (Campos et al., 1992).

2–5 A synthetase activity rose dramatically following injection with the rBoIFN-α slow release formulation. However, unlike the changes noticed in $\gamma\delta^+$ cell phenotype, levels remained high for the duration of the experiment. It is believed that 2–5 A synthetase may have beneficial effects in reducing virus replication by either substantially decreasing both host cell and viral protein synthesis, which results in cell death and therefore prevention of progeny virus release. Alternatively, viral but not host protein synthesis may be inhibited directly affecting virus survivals (McMahon and Kerr, 1983). It is now accepted that 2–5 A synthetase activity is an accurate correlate of anti-viral activity and has been described as a prognostic indicator of viral infections in the bovine respiratory tract (Bielefeldt-Ohmann et al., 1989). Since 2–5 A synthetase levels were only elevated for a short period following administration of 17 μg of soluble rBoIFN-α as compared to the slow release formulation, it is apparent that prolonged elevation of 2–5 A synthetase in the latter formulation was due to an extended release of rBoIFN-α rather than to an increase in rBoIFN-α dose. Interestingly, 2–5 A synthetase levels remained high in the presence of marginal rBoIFN-α titres in serum. This substantiates previous observations that 2–5 A synthetase is a highly sensitive indicator of IFN-α activity (Bielefeldt-Ohmann et al., 1987; Perino et al., 1990). Moreover, this observation indicates that extremely low levels of IFN-α may be required to induce maximal 2–5 A synthetase activity and that the rBoIFN-α dose of 50 mg in this slow release formulation could be reduced to a fraction of its present level.

Interferon α treatment has been described as being able to affect natural killer (NK) cell cytotoxicity (Einhorn et al., 1980) and oxygen radical production (Bielefeldt-Ohmann et al., 1987). In this study, we saw no evidence of NK or LAK augmentation as demonstrated, nor an increase in oxygen radical production was measured by the NBT reaction. These observations support our hypothesis that while the antiviral state remains high for 8 days, immunomodulation by rBoIFN-α may be extremely transient. Changes in cell trafficking were transient following injection of rBoIFN-α in the slow release formulation. Thus, for the immunoregulatory effects to be exploited, it is

possible that multiple injections will be necessary, as has been described previously (Griebel et al., 1989).

These studies have highlighted two significant findings. 2–5 A synthetase activity in PBMC increased and remained elevated following injection of a slow release formulation of rBoIFN-α for at least 8 days. An equivalent dose of IFN-α, based on the bioavailability of rBoIFN-α in this formulation, was unable to prolong the elevation of levels of this enzyme. These data imply that the antiviral effects of rBoIFN-α could be prolonged using a slow release protocol.

Acknowledgements

The authors thank the Animal Care Unit and technicians in the Immunology Program. This work was supported by CIBA-GEIGY Canada Ltd. and the Natural Sciences and Engineering Council of Canada. Published with permission of the Director as journal article number 151.

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