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Activity of polymorphonuclear (PMN) leukocytes during bovine herpes virus-1 induced respiratory disease: effect of recombinant bovine interferon alpha$_I$[1]

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Summary

Following infection of cattle with bovine herpes virus-1 there is a state of generalized immunosuppression involving various leukocytes including polymorphonuclear (PMN) leukocytes. Since the PMN is considered to be pivotal in recovery from secondary bacterial infections during bovine respiratory disease, investigations were initiated to determine PMN activity in this disease and whether interferon could modulate PMN activity. In this study, the in vivo administration of recombinant interferon alpha-$_I$[1] was shown to increase PMN functions as measured by migration/chemotaxis and generation of reactive oxygen species. This augmented activity of PMN appeared to correlate with the reduction of overall clinical disease, that is, number of sick days, lung lesions and weight loss. In the present study administration of interferon by the intranasal or intramuscular route were as effective in stimulating PMN function. Based on these studies it was concluded that the reason for improved performance of calves treated with interferon would be

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due to its immunomodulatory effects on leukocytes. Although interferon did not alter the initial suppression of PMN functions, these functions returned to normal and exceeded normal activities by 7–9 days post-infection, the time when maximal bacterial activity normally is present.

Polymorphonuclear (PMN) leucocytes; Bovine herpes virus-1 (BHV-1); Recombinant bovine interferon alpha$_I^1$ (rBoIFN-α_I^1)

Introduction

Acute bovine respiratory disease (BRD) is an economically important disease to the North American cattle industry (Babiuk et al., 1987). The etiology of this disease is highly complex involving viruses, bacteria and stress. Since BRD is multifactorial, involving many different pathogens, vaccination against individual pathogens has proven not to be very effective. In many instances, immunosuppression due to stress and viral infection are responsible for the increased susceptibility to bacterial colonization. To overcome these factors recombinant bovine interferon-alpha-$_I^1$ (rBoIFN-α_I^1) was used as an immunomodulatory agent (Babiuk et al., 1985). In this preliminary study, the treatment of calves with rBoIFN-α_I^1 prior to challenge with BHV-1 reduced the severity of clinical disease after a subsequent challenge with *Pasteurella haemolytica*. It was also shown that this increased resistance to *P. haemolytica* was at least partly due to the compound's immunomodulatory function. Interferon has been shown to enhance or suppress both the humoral and cellular immune response (Nacamura et al., 1984; Senik et al., 1979; Shalaby et al., 1984). Modulation of the immune response by interferon may depend not only upon timing and dose but also on the type of interferon used, since the different IFN species may differ in their immunoregulatory function (Babiuk et al., 1985).

In an attempt to gain further insight into the role of rBoIFN-α_I^1 as a valuable adjunct to vaccines for BRD, clinical studies using a BRD model system were conducted, in which the efficacy of the in vivo administration of rBoIFN-α_I^1 in reducing morbidity and mortality of BHV-1/*P. haemolytica* infected cattle, was evaluated. A detailed account of the clinical assessment has been published elsewhere (Babiuk et al., 1987). In this communication we report on the functional capacity of PMNs isolated from rBoIFN-α_I^1-treated animals during challenge with BHV-1/*P. haemolytica*. We chose to study the immunomodulatory role of rBoIFN-α_I^1 on PMN function since, as stated earlier, phagocytic PMNs are probably largely responsible for clearing a *P. haemolytica* infection (Babiuk et al., 1987; Walker et al., 1983). Furthermore, neutrophil products in combination with bacterial toxins are the major cause of lung injury (Slocombe et al., 1985) in BRD.

Materials and Methods

Animals

Range-bred, healthy Hereford calves aged 6–10 months and weighing 150–250 kg were used throughout the studies. All animals were seronegative for bovine herpesvirus type 1 (BHV-1) and *P. haemolytica* and neither of these agents were isolated from the animals prior to challenge. The calves were housed either in isolation or in an outdoor pen without any contact with other animals. In all cases they were fed alfalfa pellets and water ad libitum.

Virus

BHV-1 strain 108 was cultured in Georgia bovine kidney (GBK) cells as described previously (Babiuk et al., 1975, 1985). Following infection of confluent monolayers at a multiplicity of infection (MOI) of 1, cultures exhibited extensive cytopathology within 36 h, at which time they were harvested, titrated and stored at −70°C until used. This virus preparation had an infectivity of 10^7 PFU/ml and was used undiluted for aerosol challenge.

Bacteria

P. haemolytica (biotype A, serotype 1) was grown in brain heart infusion (BHI) broth containing 5% horse serum as described previously (Bielefeldt Ohmann et al., 1985). For challenge, the bacterial culture was always in the exponential phase of growth (6 h culture) at the time it was used as an aerosol and had a titre of 1–2 $\times$ 10^9 colony-forming units per ml (CFU/ml).

Recombinant bovine IFN

rBoIFN-α_I[1], produced in *Escherichia coli* by recombinant DNA technology and purified to homogeneity, was provided by CIBA-GEIGY Limited, Basel, Switzerland. The titre of the stock IFN preparation was 1.7×10^7 units/mg protein.

Experimental procedures

Calves were transported from a ranch to the research facility and rested for at least 2 days prior to initiation of the study. During this period they were subjected to a thorough clinical examination including recording of temperatures and of leukocyte cell numbers and functional activity. Analysis of the microbial flora in the nasal passages was also conducted to ensure that there had been no recent infection with pathogens. At the start of the experiment, calves were randomly divided into groups (5 – 10 animals/group) and were either treated intranasally or intramuscularly with interferon or with a placebo at the times specified (usually 48 h) prior to being challenged with BHV-1 virus, followed 4 days later with *P. haemolytica* as described previously (Bielefeldt Ohmann et al., 1985).

Clinical evaluation

The clinical evaluations were performed at the same time each day by 2 independent investigators who were uninformed about the specific treatment of the in-

dividual animals. The parameters evaluated included depression, appetite, fever, conjuctivitis, rhinitis, respiratory distress, tracheitis, pneumonia and dehydration. In each case a score of zero was assigned to healthy animals. Clinical scores of 1–4 were assigned in each category to sick animals as follows: 4, severe; 3, marked; 2, moderate; 1, mild. Total clinical scores for each animal are the sums of scores for each parameter.

Necropsy – gross pathology

Post mortem examinations were performed on animals that died or were killed during the experiments. Particular attention was paid to the nasal passages, larynx, trachea and lungs. The extent of pneumonia was assessed by a numerical scoring system as developed by Thompson et al. (1975). Briefly, each lung lobe (except the accessory lobe) was scored from 0–5 according to the amount of tissue involved. Total scores for 7 lung lobes ranged from 0 to a maximum of 35, if the whole lung was affected.

PMN isolation

Peripheral blood was collected, from the jugular vein, into sodium citrate. The citrated blood was centrifuged and the buffy coat layer removed. The polymorphonuclear neutrophilic granulocyte population was isolated from the erythrocyte pellet by hypertonic lysis of the erythrocytes as previously described (Bielefeldt Ohmann et al., 1984a).

Functional analysis of PMN population

Stock opsonized zymosan and zymosan activated serum. Stock opsonized zymosan was prepared using pre-boiled zymosan (Sigma Chemicals) in phosphate buffered saline (PBS) at a concentration of 50 mg/ml. To one ml of stock zymosan was added 3 ml of bovine serum. The zymosan/serum mixture was incubated at 37°C for 30 min, and then centrifuged at 100 × *g* for 5 min. The serum was removed and stored on ice for subsequent use in the migration, chemotaxis assays as a source of chemoattractant. The opsonized zymosan was resuspended in PBS to a final concentration of 12.5 mg/ml.

Migration. Migration of PMNs was assayed in agarose plates as previously described (Bielefeldt Ohmann et al., 1984a; Nelson et al., 1975). Briefly, PMNs were added in 10 μl volumes, at a cell concentration of 5×10^7 PMN/ml, to the inner well cut into agarose plates. The chemoattractant used was zymosan activated bovine serum, as a putative source of the potent chemotactic complement split product C_{5a}. The serum was added (10 μl) to the center well. To control for random migration, PMNs were resuspended in an equal volume of activated serum prior to being added to the outer well. The plates were then incubated at 37°C for 2 h. Following migration the plates were fixed, stained and the migration distances were measured using a calibrated grid mounted into the eye piece of a stereo-microscope. The distance of migration for PMNs suspended in the serum (random movement) was subtracted from the distance of migration of the PMNs (resus-

pended) in Hank's balanced salt solution (HBSS) towards the chemoattractant. The resulting difference was determined to be directional (chemotactic) migration.

Chemotaxis. In vitro chemotactic responses were measured as described by Gee et al. (1983) using multi-well microchemotaxis chambers (Neuroprobe, Maryland) and 5 μm polycarbonate membranes (Nucleopore, California). The chemoattractant utilized in the assays was zymosan-activated bovine serum as a putative source of the complement split product C_{5a}. 25 μl of the chemoattractant was added to the bottom wells of the chemotaxis chamber and 50 μl of the cell suspension (2×10^6 cells/ml) was placed in the top chamber wells. The chambers were incubated for 2 h in a humidified incubator at 37°C. The membranes were removed and non-migrating cells were scraped from the upper surfaces. The membranes were then fixed and stained with a modified Wrights-Geimsa stain. Following staining, the membranes were examined microscopically for migrating cells. The cell counts are presented as the mean of counts from 3 representative high powered fields (×400).

Superoxide production. Superoxide anion generation was measured in accordance with the method described by Johnson et al. (1978) and modified by Bielefeldt Ohmann et al. (1984b). Briefly, superoxide anion generation and release was measured by the superoxide dismutase (SOD) inhibitable reduction of ferricytochrome C. All samples were measured in duplicate and in suspension in a final volume of 1 ml. All tests suspensions received the substrate ferricytochrome C with or without 10 μg/ml of superoxide dismutase and 1 mg of opsonized zymosan. The samples were then incubated for 45 min at 37°C. The reaction was terminated by transferring 1 ml aliquots to an ice-bath following centrifugation. Cytochrome C reduction was monitored by a spectrophotometer at 550 nm. The OD value was then converted to nmol of superoxide/cell concentration.

Luminol enhanced chemiluminescence. Luminol enhanced chemiluminescence was carried out as described by Abramson et al. (1982). A PMN cell concentration of 1×10^7 cells/ml was found to give optimal response in the chemiluminescent assay (Lawman, unpublished data). To scintillation vials were added 5 ml of HBSS (without Phenol Red), 400 μl of opsonized zymosan and 20 μl of luminol (5 amino-2,3 dihydro-1,4-phthalazinedione) at a concentration of 275 μM. The vials were placed into a Beckman scintillation counter, set in an out of coincidence mode, and the light emissions read until an equilibrium in the background counts per minute (CPM) was observed. 0.5 ml of each PMN cell suspension was added to duplicate vials. One minute counts were taken at a 9-min time interval until such time as the light emissions returned to base-line. The results are expressed as CPM.

Results

Effect of interferon on clinical responses

Previous studies have clearly shown that rBoIFN-α_1[1] administered by the in-

Table 1

Effect of Bovine Alpha Interferon Treatment on Respiratory Disease.

Expt.	Treatment	Mean clinical score[a]	Total sick days[b]	Deaths	Mean lung score[c]	Weight loss (kg)
1	Placebo	79.0	27	6/10	12.3	ND
	10 mg BoIFN (IN)	61.8	15	2/10	3.2	ND
	10 mg BoIFN (IM)	51.2	9	2/10	3.5	ND
2	Placebo	69.3	29	6/9	13.3	7
	5 mg BoIFN (IM)	33.1	7	2/10	4.8	3.2
	10 mg BoIFN (IM)	33.9	7	3/10	6.3	4.1

[a] Mean clinical score over a 12-day observation period. Number of animals in each group was 10.

[b] Total sick days = the number of calf days that animals in a group scored 10 or more. Animals scoring 10 or more would have readily apparent signs of respiratory disease and would likely be selected for treatment in a natural disease outbreak.

[c] Based on a quantitative postmortem scoring system that estimates the volume of lung tissue affected. A score of 0 indicates a normal lung, while a score of 35 means the entire lung is involved. Surviving animals are scored 0.

tranasal route was effective in reducing the clinical responses of animals infected with BHV-1 followed 4 days later with *P. haemolytica* (Babiuk et al., 1987). These results also indicate that there is a broad range of rBoIFN-α_1[1] efficacy once a threshold of approximately 1 mg of interferon/animal is achieved (Babiuk et al., 1987). Since there was no significant difference in the clinical responses of animals given rBoIFN-α_1[1] at doses between 1 and 50 mg, a number of studies were conducted wherein rBoIFN-α_1[1] was administered either intranasally or intramuscularly at a dose of 10 mg/animal. Table 1 demonstrates that interferon is effective in reducing clinical signs, sick days, deaths and extent of lung involvement in the BRD model used in the present report. In these studies there was no significant difference between animals treated intranasally or intramuscularly indicating that either route was effective. In addition to having lower clinical scores, interferon treated animals also exhibited lower body temperatures than non-treated placebo controls (data not shown).

To determine whether rBoIFN-α_1[1] played an immunomodulatory role in vivo, a variety of leukocyte functions were measured. Emphasis, in this study, was placed on the PMNs since it is well recognized that this cell population plays a major role in the host defense against gram-negative bacterial infections. It is sufficient to point out that little or no modulation of lymphocyte function was observed in this model as a result of IFN treatment (data not given).

PMN chemotaxis and migration

PMN migration and the effect of rBoIFN-α_1[1] dose and route on that migration was measured by both migration (distance) and chemotactic response. In the model, peak suppression of both migration and chemotaxis occurred 4 days after BHV-1 infection. This is the crucial time at which the host becomes maximally susceptible to *P. haemolytica* infection. This suppression is evident in all groups and treatment

regimes investigated (Fig. 1).

In the migration and chemotaxis studies, the rBoIFN-$\alpha_I{}^1$-treated animals returned to normal levels more rapidly than did the placebo animals (Fig. 1). However, there was no significant difference between the rBoIFN-$\alpha_I{}^1$ treatment when comparing intranasal and intramuscular routes of administration or 5 mg and 10 mg doses. In chemotaxis assays the total number of PMNs responding chemotactically to activated serum (used at a final dilution of 1/25 in order to obtain peak PMV response) was measured. The rBoIFN-$\alpha_I{}^1$ groups, while showing suppres-

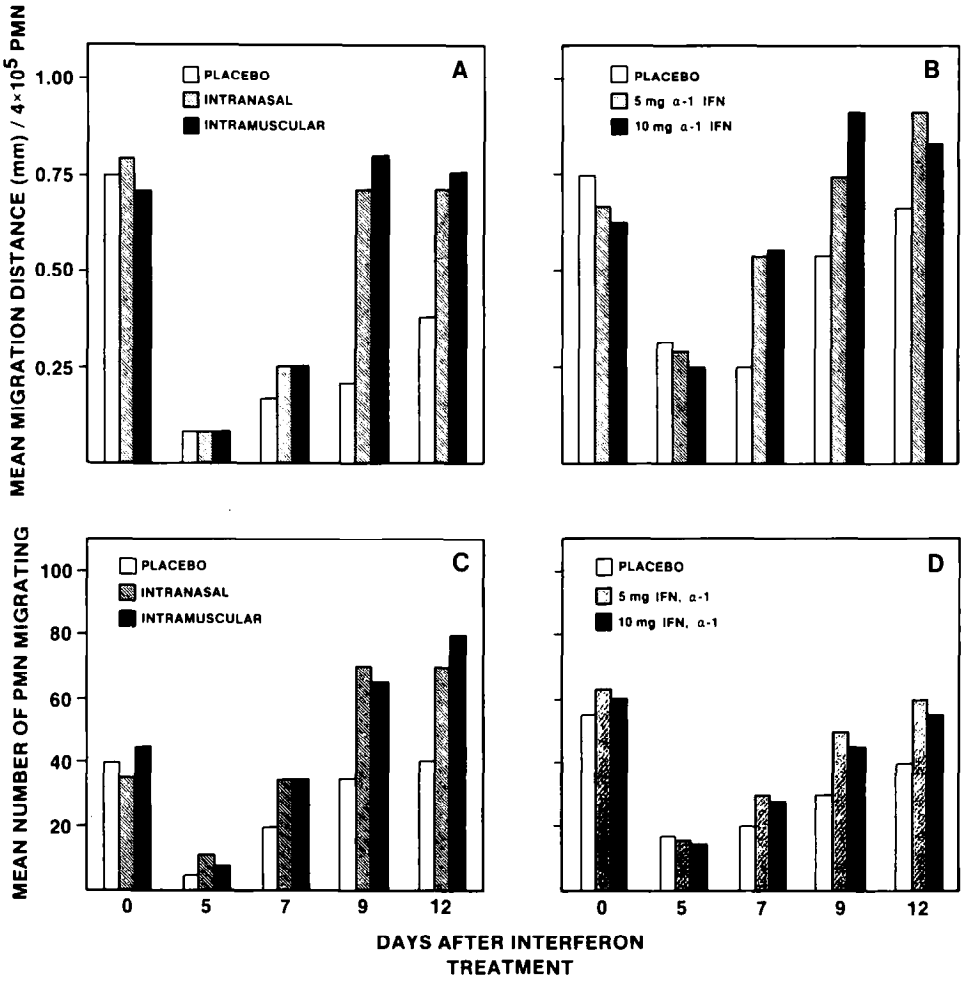

Fig. 1. Effect of BHV-1/*P. haemolytica* infection on migration and chemotaxis of polymorphonuclear leukocytes. The migration was measured under agar at various times after BHV-1 infection. The mean migration distance represents the difference between PMN response to zymosan activated bovine serum and random movement (see Materials and Methods). Chemotaxis was measured in microchemotaxis chambers. Significant differences ($P < .01$) were seen between interferon treated and placebo treated animals at days 9 and 12.

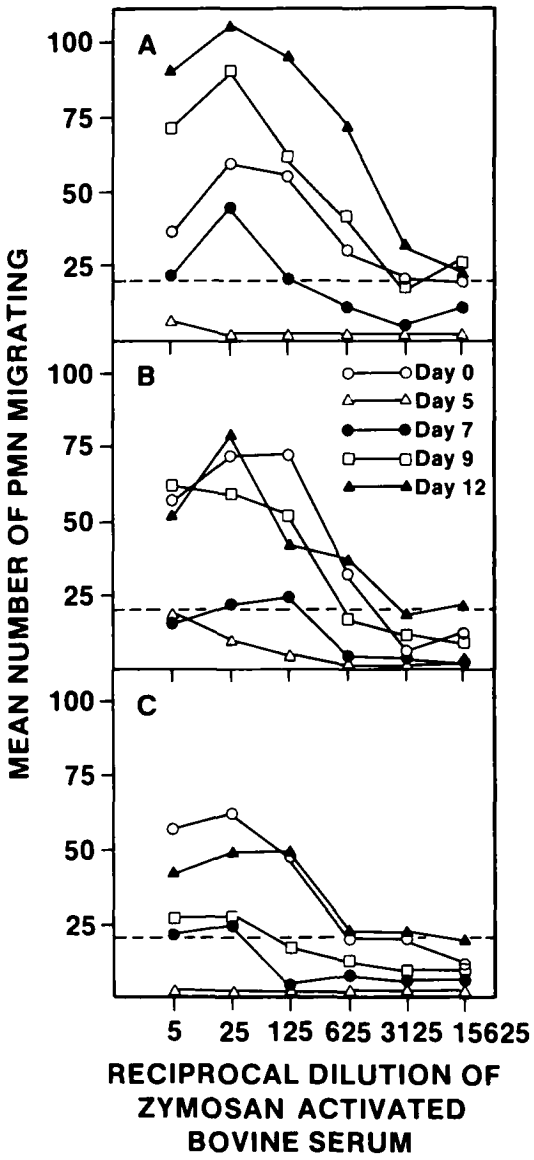

Fig. 2. Kinetics of the chemotaxis response of polymorphonulcear leukocytes from animals challenged with BHV-1/*P. haemolytica*. The interferon treatment was 48 h before BHV-1 challenge. (A) Interferon given intramuscularly. (B) Interferon given intranasally. (C) Placebo control.

sion in PMN chemotactic response (Day 5), were able to recover their chemotactic activity earlier and at increased levels than the placebo animals. In concurrence with the migration results there was no real difference between any of the rBoIFN-α_1[1] treatment groups. Fig. 2 shows a typical chemotactic response to a range of dilutions of the chemoattractant (zymosan activated bovine serum). These re-

sults are representative of individual animals in the rBoIFN-$\alpha_I{}^1$ treatment groups (intramuscular and intranasal) and the control group (Placebo). In these results the PMN chemotactic response at different times after rBoIFN-$\alpha_I{}^1$ treatment was measured using a 5-fold dilution series of bovine zymosan activated serum. The peak PMN migratory response occurred at a dilution of 1/25 of the chemoattractant regardless of the time post-infection and specific treatment regime, i.e. whether rBoIFN-$\alpha_I{}^1$ or a placebo was administered. Evident, however, was a difference in the level of the chemotactic response (number of cells migrating). In Fig. 2C, the placebo animals showed a significant suppression at Day 5 with a slow recovery of chemotactic ability. Day 12 was the only time point in which a significantly elevated chemotactic response was measured over and above the chemokinetic, or non-vectorial movement (shown by the dotted line). The animals to which rBoIFN-$\alpha_I{}^1$ was administered intramuscularly (Fig. 2A) also showed marked suppression at Day 5. However, in rBoIFN-$\alpha_I{}^1$-treated animals, the PMNs rapidly recovered their chemotactic ability such that from Day 7 through Day 12 there was a significant chemotactic response, which on Days 9 and 12 was appreciably higher than that for the Day 0 time point. A similar finding was observed for the animals which received rBoIFN-$\alpha_I{}^1$ intranasally (Fig. 2B). Again significant suppression was recorded on Day 5 with a rapid return to normal levels of PMN chemotactic function. It is interesting to note that the levels of chemokinetic movement, while slightly suppressed at Day 5 did not increase above normal background levels in any of the rBoIFN-$\alpha_I{}^1$-treated or placebo groups.

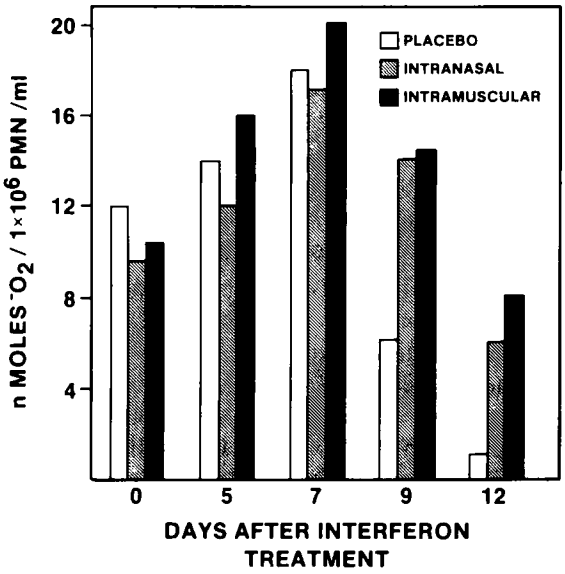

Fig. 3. Effect of BHV-1/*P. haemolytica* infection on the generation of superoxide anions by bovine polymorphonuclear leukocytes. Influence of interferon when given intramuscularly and intranasally. The interferon was administered 48 h prior to infection with BHV-1. Significant difference ($P < .01$) between interferon treated and placebo on days 5, 7, 9 and 12.

Production of reactive oxygen species

Phagocytic uptake of bacteria is a primary step in the clearance of bacteria. The killing of bacteria appears to be dependent upon the oxidative metabolism of the phagocyte and the degranulation of lysosomal constituents into the phagocytic vacuole (Chatelet, 1979). To evaluate the modulatory role of rBoIFN-$\alpha_I{}^1$ on the production of reactive oxygen species (ROS) 2 methods were employed: the generation and release of superoxide anions (O_2^-) and luminol-enhanced chemiluminescence.

The production of and release of O_2^- in the intranasal and intramuscular rBoIFN-$\alpha_I{}^1$ treated groups did not differ from those of the placebo group up to seven days after the administration of rBoIFN-$\alpha_I{}^1$ (Fig. 3). In all cases there was an increase in the ability of the PMN to respond to opsonized zymosan. Peak activity occurred between 7 and 8 days post rBoIFN-$\alpha_I{}^1$ administration, which is 24–48 h after *P. haemolytica* instillation (see Fig. 3). The greatest difference seen between the rBoIFN-$\alpha_I{}^1$ groups and the placebo controls occurred from Day 9 onwards. The PMN from the placebo controls appeared to lose their ability to respond in the production of ROS while the rBoIFN-$\alpha_I{}^1$ treated groups maintained significant levels of ROS production. There was, however, no statistical difference between the intramuscular and the intranasal rBoIFN-$\alpha_I{}^1$ groups. Although all groups increased their ability to produce O_2^- on Day 7, over that seen on day 5, only the rBoIFN-$\alpha_I{}^1$ treated groups reached pre-infection levels. At later time points, control animals continued to have reduced ability to produce O_2^-. As was seen in Fig. 3, by Day 9, the placebo group's O_2^- production declined faster than that for the rBoIFN-$\alpha_I{}^1$ groups. This basic trend was also observed in the comparison of 10 mg IFN and 5 mg rBoIFN-$\alpha_I{}^1$ (data not shown).

In studying the production of ROS by chemiluminescence a more dramatic response was observed. Fig. 4 demonstrates the chemiluminescence response of the rBoIFN-$\alpha_I{}^1$-treated animals (intramuscular and intranasal) versus the placebo group. The placebo animals while exhibiting increases in the production of ROS, their levels were much lower than those for the rBoIFN-$\alpha_I{}^1$ groups. Peak activity occurred at day 9 for all 3 groups with the highest activity occurring in the intramuscular rBoIFN-$\alpha_I{}^1$-administered group followed by the intranasal, rBoIFN-$\alpha_I{}^1$-administered group.

Discussion

The in vitro treatment of bovine phagocytic cells with rBoIFN-$\alpha_I{}^1$ has shown that pre-treatment with rBoIFN-$\alpha_I{}^1$ enhanced such functions as phagocytosis, Fc receptor expression, production of ROS and decreased both random and directional migration (Bielefeldt Ohmann et al., 1984a). The observation that in vivo interferon treatment also enhanced PMN function at the critical time of the disease process, suggests that interferon had an influence on the eventual outcome of this disease. Whether the in vivo effects of rBoIFN-$\alpha_I{}^1$ on PMNs was direct, similar to those seen following in vitro treatment, or indirect via its effect on the production

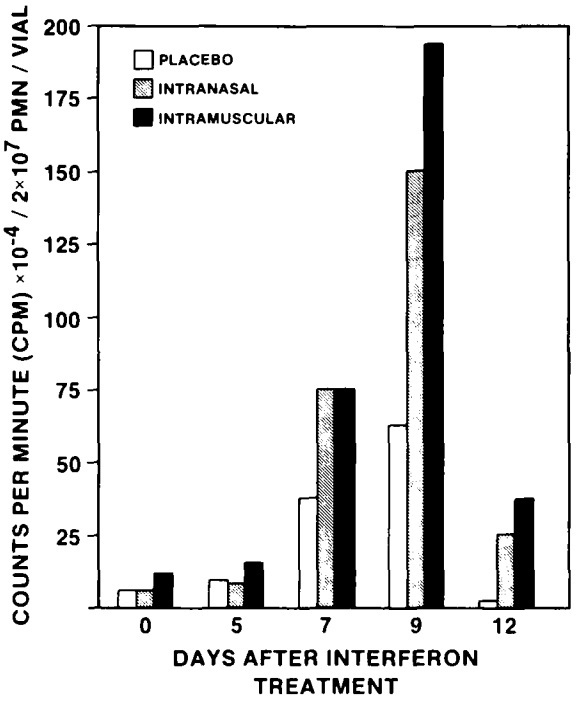

Fig. 4. Effect of BHV-1/*P. haemolytica* infection on luminol-enhanced chemiluminescence by polymorphonuclear leukocytes induced by opsonized zymosan. Influence of interferon given intramuscularly or intranasally 48 h prior to infection with BHV-1. Significant difference ($P < .01$) between interferon treated groups and placebo on days 7, 9 and 12.

of other lymphokines by cells of the lymphoreticular system remains to be determined. Furthermore, whether PMNs themselves are truly the cell that is modulated by BoIFN and is responsible for the enhanced protection, will require PMN depletion and transfer studies. These experiments are presently comtemplated, but are difficult to do in outbred species.

Exposure to pathogens generally occurs within a few days of entry of cattle into feedlots. Since animals are handled upon entry into feedlots, generally before massive exposure to viruses, the rationale for treatment of animals with interferon prior to virus exposure was designed to simulate what would occur under natural field situation if treatment was given upon entry into feedlots. Since the intranasal route of rBoIFN administration may not always be practical in feedlot situations the intramuscular route was compared with that of the intranasal route previously shown to be effective if given at 10 mg/animal (Babiuk et al., 1985). This route of administration is much more practical and economical.

We used the production of ROS as a measure of both phagocytic and bacteriocidal potential of the PMN population (van Furth et al., 1974). With this indicator of PMN phagocytic and bacteriocidal activity it was obvious that rBoIFN-$_1$[1] administration enhanced the ability of PMNs to undergo an oxidative respiratory

burst in response to an opsonized particle (zymosan).

The production and release of ROS is thought to be important in the intracellular and extracellular killing of bacteria. If this does not occur the bacteria grow and release cytotoxic products which kill or inactivate bovine leukocytes responsible for the killing of the bacteria. Thus, there is a need for a rapid influx of PMNs into the lungs early in the disease so as to eliminate the bacteria before extensive replication and lung damage occurs. If the PMNs cannot kill the bacteria, these bacterial products continue to attract PMN into the lung, which can, as a result of degranulation and release of enzymes cause damage to the lung. The importance of neutrophils in acute pasteurellosis has been shown by Slocombe et al. (1985) using neutrophil depleted calves. In this experiment it was clearly demonstrated that the interaction between bacteria and neutrophils leads to pathological lesions in the lungs of calves. The fact that interferon can enhance PMN infiltration (Fig. 1) PMN activity and superoxide release (Figs. 3 and 4) suggests that interferon reduces morbidity and mortality in this model by allowing rapid elimination of the bacteria from the lung of pasteurella infected animals before they can cause extensive damage, and thus, pasteurellosis is dramatically reduced.

The observations presented here give an indication that rBoIFN-α_1[1] therapy may be useful in the prophylactic treatment of BRD. It must also be emphasized that under experimental conditions we were unable to totally prevent morbidity and mortality but were able to reduce the severity of disease and that this reduction in disease was probably due to a positive modulation of leukocyte function, so that these effector cells respond more rapidly in the clearance of bacteria.

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