BIOLOGICAL RESPONSE OF BOVINE ALVEOLAR MACROPHAGES TO RECOMBINANT BOVINE INTERFERON ALPHA-I1

BY

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

INTRODUCTION AND REVIEW OF THE LITERATURE

The bovine alveolar macrophage (BAM) plays an important role in the primary line of defense against extraneous microorganisms such as those involved in the bovine respiratory disease (BRD) complex. Prophylactic and therapeutic properties interferon (IFN) have been considered for control of BRD. IFNs have been reported to have numerous effects on the bovine immune system including functional alterations of BAM. Response of murine macrophages to IFN has been monitored by assaying 2',5'-oligoadenylate synthetase activity (2',5'-OAS). 2',5'-OAS, one of the enzymes induced by IFN, has been shown to have antiviral and immunomodulatory effects on murine macrophages. The 2',5'-OAS assay is used to measure cellular response to IFN and has been reported to be more sensitive, rapid, and reliable than direct assay of serum IFN levels. For the reasons cited above, a decision was made to study the biological response of BAM to rBoIFN-α1 by assay of 2',5'-OAS activity *in vitro* as well as *in vivo* and viral yield reduction *in vitro* to gain better understanding of the effects of IFN on the bovine immune system and the mechanism of IFN action on BAM.

BOVINE RESPIRATORY DISEASE

Bovine respiratory diseases represent a disease complex involving stressors and viruses acting separately or together. The result of these

interactions is suppression of lung defense mechanisms which leads to predisposition to bacterial pneumonia (52). BRD has been divided into three clinical entities: enzootic pneumonia of calves, atypical interstitial pneumonia, and shipping fever complex (38). Enzootic pneumonia of calves is generally an infectious disease occurring mainly in housed calves, whereas, atypical interstitial pneumonia is a noninfectious disease of adult cattle (71). Shipping fever encompasses the acute pneumonic conditions of cattle that involve an undetermined multifactorial etiology (38,72) and are commonly associated with Pasteurella haemolytica.

BRD is responsible for major economic losses to cattle producers each year. Losses are due not only to death but also to treatment costs, preventive measures, and decreased production (72). Jensen et al. (27) reported respiratory tract diseases were responsible for 75% of illness and 64% of deaths in a survey of 407,000 yearling feedlot cattle over a one year period. Lillie (38) estimated from 40 to 80% of all cattle disease involves the respiratory system, and in Canada alone losses to the cattle industry run into millions of dollars annually.

Viruses most often associated with the infectious etiology of BRD include bovine herpesvirus-1 (BHV-1), bovine viral diarrhea virus (BVD), parainfluenza-3 virus (PI-3V), bovine respiratory syncytial virus (BRSV), bovine herpesvirus-4 (BHV-4), bovine adenoviruses (BAV), bovine rhinoviruses, bovine enteroviruses, reoviruses, and malignant catarrhal fever virus (MCF) (48). Severe respiratory disease may result due to increased susceptibility to bacterial infection of the respiratory tract. Secondary bacterial infections are predominantly by Pasteurella haemolytica or Pasteurella multocida (17). The viruses can infect an

animal, alter host defenses, and cause tissue damage. The damaged tissue provides a favorable environment for enhanced bacterial colonization, growth, and microcolony formation. The virus alone or in combination with bacteria, can also initiate the inflammatory process contributing damage to the respiratory system. Viral-bacterial synergistic interactions in respiratory infections have been extensively reviewed (1).

Challenge experiments provide evidence that viruses play an important role in predisposition to bacterial pneumonia. Jericho and Langford (29) produced pneumonia in calves exposed first to an aerosol of BHV-1 and second to an aerosol of P. haemolytica. An interval of at least four days between the virus and bacteria aerosolization was required to produce pneumonia. Excessive purulent pneumonia was produced in calves exposed to aerosols of PI-3V followed by challenge with aerosols of P. haemolytica three to ten days after virus exposure (28). Corstvet and Panciera (11) observed clinical signs of elevated temperature and respiratory distress in calves exposed to aerosols of P. haemolytica 12 days after intratracheal inoculation of BVD.

The BRD viruses are responsible for a combination of activities affecting the antiviral defense mechanisms of the lung. These activities include: impairment of mucocillary clearance; suppression of phagocytic cell function; and interference with lymphocyte function (1). BAM are permissive in culture for BHV-1 (16,62), BVD, PI-3V, and BRSV (62). Infection of BAM with PI-3V reduces their cytotoxic activity (45). BHV-1 infected BAM have reduced Fc receptors, Fc mediated phagocytosis, complement (C3b) receptor activity, and participation in antibody-dependent cell cytotoxicity (15). Bielefeldt, Ohmann, and Babiuk (7) reported depressed chemotaxis of polymorphonuclear (PMN) neutrophils and depres-

sed natural cytotoxicity and mitogen response in peripheral blood leukocytes (PBL) of cattle with experimentally induced fibrinous pneumonia. In addition, PMN superoxide anion production was transiently increased and interleukin-2 (IL-2) production was slightly decreased in these cattle.

The alveolar macrophage (AM) is considered to play an integral part in the primary defense of the lung in face of viral and/or bacterial invasion (22). Phagocytosis by BAMs is important in the nonspecific primary line of defense against BRD (69). AMs aid in the specific immune response by processing and presenting antigen to T-lymphocytes and by releasing chemotactic agents which can enhance the immune response (33). Resulting enhanced bacterial growth due to viral alteration of lung defense mechanisms, as in altered BAM function, supports the need for control of the viruses. One substance that has potential value for control of BRD is IFN.

INTERFERON

Interferon was discovered and named by Isaacs and Lindenmann in 1957 in an experiment in which allantoic membrane pieces, pre-exposed to inactivated influenza virus, released a substance capable of protecting fresh pieces of membrane against homo- or heterotypic viruses (25). Interferons are (glyco)proteins produced in virus infected cells or in cells stimulated by other interferon-inducing agents. There are three types of IFN, alpha (IFN-α), beta (IFN-β), and gamma (IFN-γ), varying in their site of production and physicochemical characteristics (30). IFN-α is produced mainly in leukocytes, IFN-β is predominantly produced in fibroblasts, and IFN-γ is produced by stimulated antigen-specific T-

lymphocytes and is also known as class II or immune IFN (30). The bovine IFN(BoIFN) system consists of two classes of BoIFN-α, class 1 (BoIFN-α1) containing 10 - 12 subtypes and class 2 (BoIFN-α2) containing 15 - 20 subtypes (9). There is one class of BoIFN-β with five subtypes and only one class and type of BoIFN-γ.

There are three types of IFN production. Type I IFN production is due to the interaction of an IFN-producing cell and an inducer. This interaction is considered the classical induction of IFN and involves inducers of varying strength including: inducers containing nucleic acid; RNA and DNA animal virus; double-stranded RNA of normal cells; synthetic double-stranded RNA; unicellular agents; microbial and cellular products; synthetic chemical compounds; and low molecular weight compounds. Type II IFN production occurs in lymphocytes activated by cell-membrane active substances or by immune induction. Type III production of IFN occurs spontaneously in normal or abnormal cells without obvious induction (24).

Several in vitro and in vivo experiments on IFN induction by various inducers have been performed in the bovine system. IFN production was induced in fetal bovine kidney cell cultures infected with unirradiated and an irradiated strain of PI-3V (50). High levels of circulating interferons were also induced in calves intravenously administered with BHV-1 virus (49). Fulton and Rosenquist (20) reported increased IFN concentrations in cultures of bovine fetal spleen cells, alveolar macrophages from adult cattle, and adult PBL in suspension which were treated with BHV-1 virus. They also demonstrated IFN production in bovine PBL treated with phytohemagglutinin (PHA). High serum IFN titers have also been detected in the early stage of infection in calves infected with an

aerosol of BRSV (14). Letchworth and Carmichael (37) reported IFN production *in vitro* by bovine peripheral blood monocytes in response to bovine herpesvirus-2 (BHV-2). In addition to viral IFN inducers, other investigators have shown increased IFN production in the bovine due to synthetic and/or natural dsRNA treatment *in vitro* of bovine cells or *in vivo* treatment of calves (34,47,51).

Interferons are responsible for antiproliferative, immunomodulatory, and antiviral effects in the bovine as well as in other animals. The antiproliferative effects of BoIFN in the bovine system have not received as much attention as antiviral and immunomodulatory effects. Czarniecki et al. (13) reported on IFN-induced inhibition of cell growth in MDBK cell cultures. In their study, MDBK cell cultures were treated with BoIFN-α1, β 2, γ , and human interferon alpha 2 (HuIFN-α_2), and then observed seven days later for viable cell numbers. BoIFN-γ exhibited the greatest antiproliferative activity of the IFNs tested. There remain unknown aspects of the antiproliferative effects of IFN in the bovine. However, the focus of attention on IFN in the bovine will probably remain on the antiviral and immunomodulatory functions. These two functions relate more to the potential prophylactic and therapeutic use of IFN in BRD.

Both the increased availability of recombinant DNA-derived BoIFN, and the conflicting *in vitro* and *in vivo* results on the antiviral activity of IFN in cattle, have stimulated interest in the immunomodulatory effects of IFN in the bovine. Most experiments on bovine immunomodulation have been performed *in vitro*, but limited *in vivo* studies have been conducted to investigate consistency with *in vitro* results. The immune cells most often associated with primary lung defense are alveolar

macrophages, monocytes, and polymorphonuclear neutrophils (43). These cells are mainly involved with the nonspecific cell-mediated immune response in reference to primary lung defense. Treatment of these cells with BoIFN-α1 has the following effects: 1) increased bacterial uptake by all three cells; 2) increased Fc receptors on alveolar macrophages; 3) migration inhibition of monocytes and polymorphonuclear granulocytes; and 4) decreased lysozyme synthesis and/or increased secretion, increased hydrogen peroxide production, and decreased superoxide anion release by alveolar macrophages and polymorphonuclear granulocytes (5). BoIFN-γ has even a greater effect on the leukocyte function than BoIFN-α (4).

BoIFNs also modulate the natural cell-mediated cytotoxicity (NC) of the bovine. Bielefeldt, Ohmann, and Babiuk (6) reported in vitro and in vivo increased NC activity in healthy cattle. Lymphocyte proliferation ws suppressed 24 hours after PBL were treated in vitro with BoIFN-α and -γ (4). Such suppression was reversible by addition of exogenous IL-2 and was due to BoIFN induction of suppressor cells (4).

In vitro and in vivo BoIFN-γ treatment of BAMs enhanced major histocompatibility complex class II (Mhc class II) (Ia) antigen expression (8). However, in vivo, but not in vitro, class II antigen expression was enhanced in BAM of healthy cattle treated with BoIFN-α1. BAM class II antigen expression is involved in antigen presentation to class II restricted antigen-specific T-lymphocytes.

Initial studies on BoIFN dealt mainly with antiviral effects. Numerous in vitro and some in vivo studies have been performed which examined antiviral activities of BoIFN against BRD viruses (2,3,12,18,19,21,63). All three types of BoIFN (α, β, and γ) have exhibited antiviral activity

to a certain degree (9). Sensitivity of viruses to IFN-induced viral inhibition varies (13,18,19,21). Of the three most commonly isolated BRD associated viruses (BHV-1, PI-3V, and BVD), BHV-1 is the least sensitive to the antiviral effects of IFN (13,18,21). BoIFN-α and BoIFN-β have greater antiviral activity than BoIFN-γ; however, BoIFN-γ has the greatest antiproliferative activity (13).

Varied viral sensitivity and the various roles of the three BoIFNs may explain the difference in results of some in vitro and in vivo BoIFN studies. Calves treated with an aerosol or intramuscular injection of BoIFN-α1 48 hours prior to challenge with BHV-1 were less susceptible to a subsequent P. haemolytica challenge than calves that did not receive IFN (2,3). Decreased severity of clinical illness was not attributed to direct antiviral effects of BoIFN-α1. Instead, alterations in leukocyte functions were suggested to be responsible for the reduced severity of clinical illness (2).

Viral induced BoIFN has also been reported to protect against subsequent challenge exposure. Intranasal inoculation of calves with a BHV-1 vaccinal strain provided protection against challenge exposure with PI-3V (12). The mean PI-3V titers in nasal secretions were reduced in BHV-1 virus-inoculated calves as compared to control calves. In addition, virus excretion was not detected in PI-3V low dose ($10^{5.3}$ $TCID_{50}$) challenged BHV-1 inoculated calves as compared to control calves.

2',5'-OLIGOADENYLATE SYNTHETASE

IFN is responsible for the production of several substances in a cell. Two of the most investigated are 2',5'-oligoadenylate synthetase (2',5'-OAS) and protein kinase (58). The current investigations are

focused on 2',5'-OAS. Kerr et al. (32) published the initial report of 2',5'-OAS in 1977. All three types of IFN are capable of inducing 2',5'-OAS (30). IFN induces the dsRNA dependent 2',5'-OAS enzyme which polymerizes ATP into a series of 2',5'-phosphodiester bond linked oligonucleotides (31). Nanomolar concentrations of these oligoadenylates (2',5'-A) activate a 2',5'-A dependent latent endoribonuclease (56) which cleaves mRNA and rRNA resulting in inhibition of mRNA translation. Inhibition of viral mRNA translation is one of the mechanisms for the antiviral state observed in IFN treated cells. 2',5'-A is also produced in uninfected cells which are treated with IFN (10,35,59,61). Increased 2',5'-OAS activity also occur during cell maturation and differentiation which suggests a possible role in controlling these cellular processes (26,60). The majority of investigations on the role of 2',5'-OAS, including its value as a marker for IFN induction and acute viral infections, have been performed in the human and murine systems.

Schattner et al. (55) reported increased 2',5'-OAS activity in mononuclear cells and granulocytes of human patients given intramuscular injections of IFN-α. The increased enzyme levels remained elevated for at least 24 hours after IFN injection compared to serum IFN levels which decreased by 24 hours. Merritt et al. (42) observed a similar response in 2',5'-OAS levels of PBML cells from human patients injected with IFN-α. In addition, Merritt et al. (41) found the magnitude and persistence of the enzyme response to be a function of IFN dose. Enzyme activity was not related to the duration of treatment, number of injections, or the route of administration. IFN assays measuring antiviral activity are more time consuming and less sensitive than 2',5'-OAS assay (41,53). The assay of 2',5'-OAS in human peripheral white blood cells to monitor

IFN therapy has been suggested. Assay of 2',5'-OAS in peripheral blood mononuclear leukocyte (PBML) cells of patients has also been suggested as a diagnostic aid for patients with acute viral diseases and autoimmune diseases (53). PBML cells of patients receiving intramuscular and intravenous injections of HuIFN-α2a exhibited a parallel response of increased vesicular stomatitis virus (VSV) yield reduction and 2',5'-OAS induction (70). Both responses were related to HuIFN-α2a dose. Three different investigations reported varied baseline 2',5'-OAS concentrations among the patients tested (42,53,55,70).

Results similar to those in the human 2',5'-OAS system have been reported for the murine system. VSV or Sindbis virus (SBV) infection of mice elicited a 4 - 7 fold increase in 2',5'-OAS (54). Enzyme activity in murine PBML cells remained elevated for approximately one week before returning to baseline levels. Although 2',5'-OAS activity was similar in the mice infected with one or the other virus, the changes in serum IFN levels varied between the two viruses. These findings suggest assay of 2',5'-OAS for detection of viral infections and/or induction of endogenous interferon. Mice injected with antibody to mouse IFN-α/β had decreased 2',5'-OAS concentrations in resident peritoneal macrophages (23). Decreased enzyme levels render macrophages permissive for VSV. Peritoneal macrophages from 1-week old mice had lower basal enzyme levels than 4 to 8-week old mice and were more permissive to VSV infection. However, peritoneal macrophages from 4 to 6-month old mice became increasingly permissive to VSV (23). Gresser et al. (23) reported an inverse correlation between 2',5'-OAS in the peritoneal macrophages and their permissiveness to VSV. Murine peritoneal macrophages, cultured *in vitro* for 3 to 5 days, became permissive to VSV and encephalomyocarditis

virus (EMCV) (46). The loss of the antiviral state paralleled a significant decrease in intracellular 2',5'-OAS activity. Vogel and Fertsch (66) recently reported low levels of 2',5'-OAS and increased susceptibility to viral infection in macrophages of mice deficient in their ability to respond to endotoxin, an IFN inducer. Induction of 2',5'-A dependent endoribonuclease has also been observed in guinea pig peritoneal macrophages treated with mouse IFN-β (68). The induction of 2',5'-OAS in the guinea pig system is age-related (67). Peritoneal macrophages from older guinea pigs have 17% less synthetase activity than those from young guinea pigs.

Results from 2',5'-OAS studies in the bovine system have been similar to those previously discussed. Georgia bovine kidney cells (GBK) treated with HuIFN responded with increased 2',5'-OAS levels (64). GBK cells did not respond as well as human HEp2 cells. The majority of bovine 2',5'-OAS activity occurred in the nucleus as opposed to cytoplasmic activity in HEp2 cells (64).

In vitro cultures of lymphocytes from calves previously administered HuIFN-α2 im and then challenged with vaccinia virus, had elevated 2',5'-OAS levels when treated in vitro with HuIFN-α2 (65). These calves were also protected against vaccinia infection. In the same report, 2',5'-OAS was induced in vivo in lymphocytes of calves treated with IFN and infected with BHV-1. Short and Fulton (57) reported in vitro and in vivo studies which indicated increased 2',5'-OAS activity in MDBK cells treated with BoIFN and PBML of cattle injected with BoIFN-αI1 or modified live virus vaccine (BHV-1, BVD, PI-3V). The increase in 2',5'-OAS activity was reported to be proportional to both the concentration of IFN in the cell culture medium of the MDBK cells and the amount of cell-

ular extract assayed. Elevated 2',5'-OAS activity, in PBML of the cattle inoculated with BHV-1/BVD/PI-3 modified live virus vaccine, persisted at least three days postvaccination. Enzyme levels in cattle injected with BoIFN reacted in a similar manner. Most bovine studies on the 2',5'-OAS system deal with the classical mechanism of 2',5'-OAS activity involving blockage of RNA viral protein synthesis. However, one in vitro study using bovine turbinate (BTu) cells pretreated with HuIFN-α2a and challenged with PI-3V reported virus yield reduction without complete blockage of viral protein synthesis (44). That report suggested that the HuIFN-α2a affects glycoprotein synthesis and morphogenesis of PI-3V and, as a result, the antiviral state inhibited release of viral particles from the IFN treated cells.

2',5'-OAS activity has also been related to growth rate, cell differentiation, and phagocytic activity (36,39,40,60). Subconfluent densities of murine JLS-V9R cells were grown to a confluent, stationary phase. Cell growth rate was inhibited after reaching confluency at 50 h and cell division ceased after 100 h in culture. An increase in 2',5'-OAS between 50 and 100 h coincided with a substantial decrease in ^{3}H-thymidine incorporation into cellular DNA (61).

Undifferentiated (embryonal carcinoma) and differentiated (teratocarcinoma) cells differed in sensitivity to IFN-inhibition of cell growth (60). IFN had little or no effect on the growth of undifferentiated cells, but growth of differentiated cells was significantly inhibited. Embryonal carcinoma cells were also resistant to the antiviral activity of IFN when challenged with VSV, EMCV, and SBV. In contrast, IFN treated teratocarcinoma cells were protected against these viruses (60). Levels of 2',5'-A dependent endoribonuclease correlated with IFN

sensitivity in the two cell types (60). The IFN-sensitive teratocarcinoma cells contained greater amounts of the nuclease. Krause et al. (36) also found expression of 2',5'-A dependent endoribonuclease to be greatly enhanced during cell differentiation.

Immunomodulatory effects of 2',5'-A have been reported in macrophages of rabbits, mice, guinea pigs, and humans (40). These macrophages were treated with various concentrations of 2',5'-A and then allowed to phagocytose Candida albicans. Increased phagocytic activity was observed in the macrophages from each species tested (40).

RESEARCH OBJECTIVES

IFNs are currently being investigated for prophylactic and therapeutic use in BRD. The effects of IFN on BAM have been reported. However, the mechanism of IFN action responsible for viral yield reduction and other functional alterations of BAM has not been completely resolved. Recent investigations have shown monitoring of 2',5'-OAS activity to be useful as a marker for response to IFN and acute viral infections. The purpose of this study was: (1) to determine the reliability of monitoring 2',5'-OAS activity of BAM (in culture and in vivo) as a marker for IFN induction; and (2) to determine a possible mechanism of IFN action responsible for viral yield reduction and other functional changes in BAMs.

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CHAPTER II

2',5'-OLIGOADENYLATE SYNTHETASE INDUCTION AND VIRUS YIELD REDUCTION IN RECOMBINANT DNA-DERIVED BOVINE INTERFERON ALPHA-I1 TREATED BOVINE ALVEOLAR MACROPHAGES

The biological response of recombinant DNA-derived bovine interferon alpha (BoIFN-αI1) on bovine alveolar macrophages (BAM) was examined by measuring viral yield reduction and 2',5'-oligoadenylate synthetase (2',5'-OAS) production by these IFN treated cells. IFN pretreatment of BAM reduced viral yield in parainfluenza-3 virus (PI-3V) challenged cultures compared to controls. Treatment of BAM with IFN also resulted in increased 2',5'-OAS activity. The increased 2',5'-OAS activity indicates a possible mechanism of IFN action in the bovine system that may be responsible for viral yield reduction and other functional changes in BAM.

INTRODUCTION

Bovine respiratory diseases (BRD) are a major concern of the cattle industry due to the economic impact on the producer (20). The multifactorial etiology of BRD involves complex interactions between stressors and viruses acting separately or together. Viruses are responsible for a combination of activities affecting the antibacterial defense mechanisms

of the lung: impairment of mucocillary clearance; suppression of phagocytic cell function; and interference with lymphocyte function. The result being suppression of lung defense mechanisms which leads to predisposition to bacterial pneumonia (27). The enhancement of bacterial growth due to viral infection emphasizes the need for controlling both bacteria and viruses. One substance that has potential for controlling viruses and enhancing other bovine defense mechanisms (immunomodulation) in BRD is interferon (IFN).

IFNs are (glyco)proteins induced in a cell by viral infection, double-stranded RNA, or other IFN-inducing agents. There are three types of interferon, alpha (α), beta (β), and gamma (γ), varying in their site of production and physicochemical and antigenic characteristics. IFNs are responsible for antiproliferative, antiviral, and immunomodulatory effects on cells. Excellent reviews of interferons (5,15) describing their properties, actions, and potential prophylactic/therapeutic value are available. Antiviral effects of bovine and other homologous IFNs have been reported against different viral families (11,12,14). Studies have also been conducted on the immunomodulatory effects of bovine and other homologous IFNs on their respective species (6,22,23,35). Recent attention on the use of rBoIFN-αI1 as a prophylactic agent for BRD has mainly focused on its antiviral effects.

The antiviral activity of IFN includes the induction of an increase in two enzymes, 2',5'oligoadenylate synthetase (2',5'-OAS) and protein kinase. All three types of IFN are capable of inducing the enzyme 2',5'-OAS discovered by Kerr et al. (16). 2',5'-OAS synthesizes 2',5'-oligoadenylates (2',5'-A) that activates a 2',5'-A dependent RNase which degrades mRNA and rRNA, thus inhibiting virus growth (8). The enzyme

2',5'-OAS has been suggested for use as a marker of IFN action (7,21,30, 31,32,33,36,38) and viral infection (28,29,31). Merritt et al. reported increased 2',5'-OAS activity in human peripheral mononuclear cells after injection of IFN into healthy individuals and patients (21). Increased 2',5'-OAS activity was also observed in mouse peripheral blood mononuclear cells (PBMC) following ip injection of either vesicular stomatitis virus (VSV) or Simbis virus (SBV) (29). Increased 2',5'-OAS activity has also been observed in macrophages treated with IFN in vitro (36,37). Decreased activity of 2',5'-OAS in macrophages has also been related to loss of the antiviral state (26) and increased susceptibility to virus infection (13,34). Vogel and Fertsch reported macrophages from C3H/HeJ mouse strain, bearing an autosomal gene defect diminishing their ability to respond to endotoxin, were more permissive to VSV infection and exhibited low basal concentrations of 2',5'-OAS as compared to non-permissive fully endotoxin responsive mice (34). Gresser demonstrated an inverse correlation between the concentration of 2',5'-OAS and permissiveness for VSV infection in peritoneal macrophages of mice injected with antibody to mouse IFN alpha/beta (13). In addition to the classical antiviral mechanism of 2',5'-OAS, 2',5'-A has been associated with increased phagocytosis in macrophages (18,19).

Prior studies on 2',5'-OAS as related to IFN and the antiviral state have mainly focused on humans and murine species. Recent studies on the characterization of 2',5'-OAS in the bovine system (31,32,33) have provided new means of studying the IFN response in cattle. Peripheral blood mononuclear leukocytes (PBML) of calves inoculated with IBR/BVD/PI-3V modified live virus vaccine and from calves injected with IFN have shown increased 2',5'-OAS activity (31). Many studies have been

performed on functional alterations of BAMs due to IFN (1,2,3,4); however, the mechanism of IFN action on the BAMs has not been completely resolved. The BAMs are considered to be important in primary cellular defense against extraneous microorganisms (5) such as those involved in BRD; therefore, it would be advantageous to understand the mechanism of IFN action on BAM. In the present study, we measured the biological response of BAM to rBoIFN-αI1 by viral yield reduction and increased 2',5'-OAS activity.

MATERIALS AND METHODS

Animals. Three crossbred calves, weighing approximately 475 kg each, were purchased from commercial sources and were used as BAM donors. The calves were maintained on free choice hay with grain supplement.

Collection and maintenance of BAM. BAM were collected by bronchopulmonary lavage as described by Corstvet et al. (9). The lavaged cells were filtered through four layers of sterile gauze and centrifuged at 250 x g at 10°C for 10 min. The supernate was discarded and pellets resuspended in cold (40°C) Tris-buffered ammonium chloride (0.16 M NH_4Cl and 0.17 M Tris-HCl pH 7.65) to lyse contaminating RBC. The BAM were washed twice with 1X Modified Hanks Balanced Salt Solution (HBSS) without calcium, magnesium, and phenol red. Washed cells were resuspended in maintenance media consisting of RPMI 1640 (Gibco) without phenol red, with 20% fetal bovine serum (FBS), and 50 ug/ml gentamicin. BAM were seeded in 15 ml conical polystyrene centrifuge tubes for use in yield reduction and 2',5'-OAS studies. Non-specific esterase stain (17) and

cellular morphology were used to identify BAM as the predominate cell type.

Virus. PI-3V was grown in Madin Darby bovine kidney cells (MDBK), and titered by 24-well plate plaque assay on MDBK cells (10).

Interferon. IFN used in this study was recombinant DNA-derived BoIFN- I1 (Ciba-Geigy). IFN titer (4.142×10^8 U/ml) was determined by 24-well plate plaque reduction on MDBK monolayers challenged with VSV (10).

Yield reduction for antiviral activity. In three separate experiments, BAM were collected from the three animals and were seeded in 0.1 ml volumes in 15 ml conical polystyrene centrifuge tubes (5×10^5 cells/tube). The BAM were incubated for 3 h at 37°C in 5% CO_2, and then pretreated with 10,000 IFN units for 8 h under incubation conditions previously described. Control cultures not receiving IFN but infected with PI-3V were also included. After 8 h pretreatment with IFN, PI-3V was added at a M.O.I. of 0.1 and infected cells were then incubated 24 h under previously described conditions. Samples were frozen at -70°C until viral infectivity was measured by 24-well plaque assay on MDBK monolayers as described previously (10). Viral yields were expressed as PFU/0.1 ml.

Cell extracts. 2',5'-OAS activity was measured in BAM concurrently with the yield reduction experiments. BAM (1×10^6 cells in 0.2 ml maintenance media) were seeded in 15 ml polystyrene conical centrifuge tubes and incubated 3 hours at 37°C in 5% CO_2, and then pretreated with 10,000 IFN units under the same incubation conditions. Untreated control BAM cultures were also included in this experiment. At 0 h, 8 h, and 32 h post treatment, samples were processed for assay of 2',5'-OAS activ-

ity. Cells were washed once with PBS by centrifugation at 1000 x g for 1 min. The supernatants were removed and cells were then lysed by suspension in buffer containing the detergent Nonidet P-40 as described by Schattner et al. (29). After lysis buffer was added, cells were frozen at -70°C until further processing. Cells were thawed at 25°C and allowed to stand for 30 min at 4°C. Cell lysates were then transferred to polypropylene microcentrifuge tubes and centrifuged at 12,000 x g for 6 min. The cell extract (CE) was removed immediately for use in the enzyme assay.

Activity of 2',5'-oligo(A)synthetase. Enzyme activity was measured by using a modification by Short and Fulton (31) of the 2',5'-OAS assay described by Schattner et al. (29). [8-^{14}C] ATP as the substrate instead of [α-^{32}P]. Cell extracts and reaction mixture were incubated 18 h, digested with alkaline phosphatase, and then 25 ul of distilled water was added to each sample prior to freezing at -10°C. Samples were thawed at room temperature and then loaded onto Dowex 1 (Cl-) columns. Columns were eluted with 2ml of distilled water and the eluate was collected directly into scintillation vials. Columns were next eluted with 2ml of 0.01 M HCl-1.0 M NaCl which was collected into separate scintillation vials. All eluates were mixed with 15 ml of Scinti-Verse (Fisher Scientific Co.) for counting in a Pakard Tri-Carb 300 scintillation counter (Canberra Co., Laguna Hills, CA).

RESULTS

The pretreated BAM (10,000 units of BoIFN-αI1 8 h prior to challenge exposure with PI-3V at M.O.I. of 0.1), had significantly ($P < 0.001$) greater viral yield reductions than BAM not receiving IFN

(Table 1). Viral yield reductions (Log_{10} PFU/0.1 ml culture fluid) were 1.5, 0.6, and 1.1 for each animal, respectively. Although the sensitivity varied for the same animals between experiments and among animals on the same experiment, IFN treated macrophages showed consistent yield reductions compared with their control BAM. Elevated 2',5'-OAS activity was also observed in the IFN treated ($P < 0.05$) BAM as compared with untreated BAM (Fig. 1). 2',5'-OAS activity increased 80% ± 39% at the 8 h time point and 174% ± 89% at the 32 h time point in IFN treated BAM as compared to 16% ± 27% and 35% ± 31% in untreated BAM at 8 h and 32 h time points respectively. A temporal relationship occurred between percent viral yield reduction and percent increase in 2',4'-OAS activity in rBoIFN-αI1 treated BAM.

DISCUSSION

Several previous studies have shown decreased viral susceptibility (4,13,34) and altered macrophage functions (4,23,35) due to IFN. However, these studies did not address relationships between the changes in susceptibility and function with respect to possible molecular mechanisms of IFN action in the bovine system. In the present study viral yield reduction was observed in IFN treated BAM challenged *in vitro* with PI-3V. 2',5'-OAS levels also increased in BAM treated with IFN. These results suggest that 2',5'-OAS may be important in the mechanism of IFN action during the antiviral state in BAMs. The mechanism may be due to either the classical 2',5'-A dependent endoribonuclease cleavage of RNA or by other products of 2',5'-OAS activity. These findings are similar to those of Wang and Wu (35) in which IFN induced *de novo* synthesis of 2',5'-A dependent endoribonuclease activity in cultured guinea pig

macrophages, and Gresser et al. (13) who reported a correlation between levels of 2',5'-OAS in mouse peritoneal macrophages and the permissiveness of the cells for VSV. A recent study by Panigrahi et al. (24) suggested that the classical mechanism of 2',5'-A activated endonuclease and protein kinase seemed unimportant in the inhibited production of infectious PI-3V in HuIFN-α2a treated bovine turbinate cells. However, 2',5'-OAS levels were not measured in their study, instead, production of viral proteins was compared in IFN-treated and nontreated cells (24). Although total inhibition of viral proteins was not observed in their system (24), this does not rule out the possibility that 2',5'-OAS or its products were involved in inhibition of PI-3V. Incomplete blockage of viral protein synthesis could be due to varied sensitivity of viral RNA to the 2',5'-A dependent endoribonuclease or other products of the 2',5'-OAS system. Even if the classical mechanism of 2',5'-A dependent endoribonuclease is not involved in the antiviral state in the BAM, functional alterations of macrophages by 2',5'-A (18) suggests the involvement of 2',5'-OAS in the mechanism of IFN action in the bovine system to be important.

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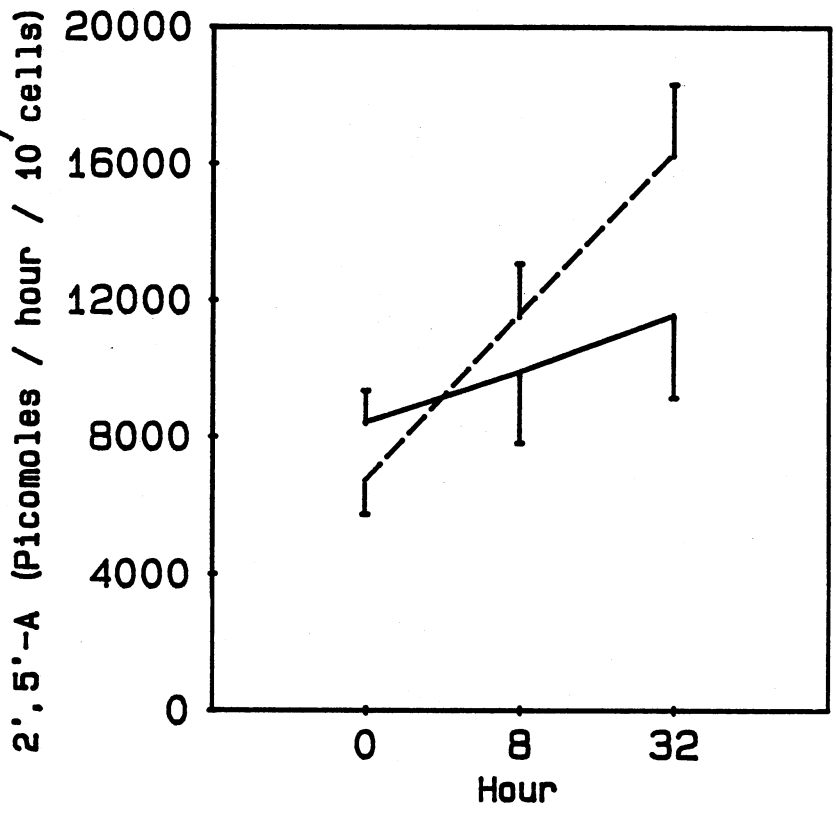
Table 1. Effect of bovine IFN on PI-3V replication in bovine alveolar macrophages

Animal #	Experiment #	Virus Yield[a]		Percent (%) reduction
		Virus control	IFN treated	
07	1	1.9×10^3	3.5×10^1	
	2	2.8×10^3	1.0×10^2	97 ± 1
	3	3.9×10^2	1.5×10^1	
11	1	1.5×10^2	4.0×10^1	
	2	1.4×10^2	6.5×10^1	73 ± 19
	3	1.9×10^2	1.5×10^1	
19	1	8.0×10^2	1.5×10^1	
	2	1.5×10^2	6.0×10^1	82 ± 19
	3	1.3×10^2	1.5×10^1	

[a]Virus yields are expressed as PFU per 0.1 ml of culture fluids. All culture fluids were from 24 h viral postchallenge collections.
[b]Percent (%) reduction is the average ± SD of % viral yield reductions for each of the 3 experiments.

Fig. 1. 2',5'-oligoadenylate synthetase activity in IFN-treated (- -) and untreated (——) cultured bovine alveolar macrophages. The results presented are the mean ± SD (n = 6) for each point.


2',5'-A (Picomoles / hour / 10^7 cells)
20000
16000
12000
8000
4000
0
0
8
32
Hour

CHAPTER III

IN VIVO INDUCTION OF 2',5'-OLIGOADENYLATE SYNTHETASE BY RECOMBINANT DNA-DERIVED BOVINE INTERFERON ALPHA-I1 IN BOVINE ALVEOLAR MACROPHAGES

2',5'-oligoadenylate synthetase (2',5'-OAS) activity was measured in bovine alveolar macrophages (BAM) and peripheral blood mononuclear leukocytes (PBML) of calves injected intramuscularly (im) with 3.6×10^6 U/kg of recombinant DNA-derived bovine alpha interferon (BoIFN-αI1). The PBML were used to monitor IFN action by measuring 2',5'-OAS activity beginning six days prior to and 24 h after IFN treatment. 2',5'-OAS activity in the PBML sharply increased 24 h after IFN treatment indicating response to IFN. BAM collected from the same calves 24 h after IFN injection also had increased 2',5'-OAS activity as compared to BAM of the same calves collected 6 days prior to treatment. The change in 2','5-OAS activity in BAM suggests the possibility of using the enzyme as a marker for IFN induction in future experiments involving IFN and its effects on macrophages and the bovine immune system.

INTRODUCTION

Interferons are (glyco)proteins produced in virus infected cells or by other inducers. There are three types of IFN, alpha (α), beta (β), and (γ), which differ in their site of production and in their physico-

chemical and antigenic characteristics. The effects of IFN on cells are antiproliferative, immunomodulating, and antiviral. The antiviral state involves two enzymes, 2',5'-OAS and protein kinase, induced by interferon. Both the human and bovine interferon systems have been extensively reviewed (3,11).

All three types of IFN are capable of inducing the enzyme 2',5'-OAS which was discovered by Kerr et al. (12). 2',5'-OAS synthesizes 2',5'-oligoadenylates (2',5'-A) which activate a 2',5'-A dependent endoribonuclease (5). The 2',5'-A dependent endoribonuclease degrades virus and cellular RNA thus inhibiting protein and DNA synthesis resulting in virus growth inhibition (5). Proietti et al. (18) found that decreased levels of 2',5'-OAS in aged mouse peritoneal macrophages paralleled the loss of the antiviral state. Other functional changes in cells have also been attributed to 2',5'-A (13,14). Liu et al. (14) reported increased phagocytic activity of rabbit, mouse, guinea pig, and human macrophages due to treatment with 2',5'-Oligo(A). 2',5'-OAS has been considered for use as a marker of IFN action (4,15,16,20-25). Witter et al. (25) showed a dose-response relationship between recombinant human IFN- 2a dose and both 2',5'-OAS activity and vesicular stomatitis virus resistance in humans.

Interferon and its effects on many bovine viruses has been investigated (2,7-9,11). Recent characterization of 2',5'-OAS in the bovine system should aid in studying the response of the bovine system to IFN (22,23). Short and Fulton (21) observed increased levels of 2',5'-OAS in PBML of calves treated with IFN and in calves administered BHV-1/BVD/PI-3V modified live virus vaccine (MLV). Their results suggest 2',5'-OAS can be used as a marker for IFN induction as is the case in humans.

Due to the emphasis on the potential of IFN use as a prophylatic in bovine respiratory disease (BRD), the measurement of 2',5'-OAS levels may be helpful in understanding the response to IFN, and possibly as a marker indicating acute viral infection (19,21).

BAM are important in primary defense of the lung against invasion of microorganisms as in BRD (3). The in vivo effects of IFN on 2',5'-OAS induction in BAM have not been studied to date; therefore, the present study measured the in vivo response of BAM to im injection of rBoIFN-αI_1 by measuring 2',5'-OAS activity in these cells.

MATERIALS AND METHODS

Animals. Crossbred calves, weighing 475 ± 19 kg each, were purchased from commercial sources and were used as BAM donors. The calves were maintained on free choice hay with grain supplement.

Collection and maintenance of BAM. BAM were collected by bronchopulmonary lavage as described by Corstvet et al. (6). The lavaged cells were filtered through four layers of sterile gauze and centrifuged 10 min at 250 x g at 10°C. The supernatant was discarded and pellets resuspended in cold (4°C) Tris-buffered ammonium chloride (0.16 m NH_4Cl and 0.17 M Tris adjusted to pH to 7.2 with HCl) to lyse contaminating erythrocytes. The BAM were washed twice with 1X Modified Hanks Balanced Salt Solution (HBSS) without calcium, magnesium, and phenol red. Washed cells were resuspended in maintenance media consisting of RPMI 1640 (Gibco) without phenol red, with 20% fetal bovine serum (FBS),and 50 ug/ml gentamicin. Cell counts and viability were calculated using a hemocytometer and trypan blue exclusion.

Interferon. The IFN used in this experiment was recombinant DNA-

derived BoIFN-αI1 (Ciba-Geigy). The IFN titers (4.142×10^8 U/ml) was determined by 24-well plate plaque reduction on Madin-Darby bovine kidney cell (MDBK) monolayers challenged with vesicular stomatitis virus VSV (7).

Cell extracts. BAM from calves administered IFN im (3.6×10^6 U/kg) and from a control calf not receiving IFN were collected and counted as previously described. BAM from all calves were also collected six days prior to IFN treatment to obtain the baseline levels of 2',5'-OAS in the BAM. A six day interval was chosen due to adverse effects on the composition of the BAM population and cell activities when shorter intervals are used (1). Duplicate samples of 5×10^5 BAM from each calf and from both collection times were placed in microcentrifuge tubes. Cells were washed once with PBS, centrifuged at 1000 x g for 1 min. The cell pellets were resuspended in lysis buffer containing the detergent Nonidet P-40 as described by Schattner et al. (19) and then frozen at -70°C until further processing. Frozen lysates were thawed at 25°C and allowed to incubate at 4°C for 30 min to complete the lysis. Lysates were then centrifuged at 12,000 x g for 6 min to remove cell debris. The cell extracts were removed immediately for use in the enzyme assay.

PBML were collected using Sepracell-MN (Sepratech Company, Oklahoma City, OK). Four ml of heparinized whole blood were added to 5.5 ml of Sepracell-MN and centrifuged at 1600 x g for 10 min in an IEC table top centrifuge with a fixed angle rotor. The top 2.5 ml were removed and added to 15 ml conical polystyrene tubes and washed 2X with PBS for 1 min at 1500 x g. Cells were resuspended in PBS and counted on a hemocytometer. PBML (1×10^6 cells in 0.2 ml maintenance media) were added to

microcentrifuge tubes and processed as described above for use in 2',5'-OAS assay.

Activity of 2',5'-OAS. Enzyme activity was measured using a modification by Short and Fulton (21) of the 2',5'-OAS assay system of Schattner et al. (19) using [8-^{14}C]ATP as the substrate in the reaction mixture. 2',5'-OAS was purified by mixing cell extract with agarose poly(I):poly(C) beads and washing with buffer. Reaction mixture and washed beads were combined and incubated at 30°C for 18 h. Alkaline phosphate was then added, temperature was increased to 37°C, and incubation was continued for 1 h. After addition of 25 ml of distilled water, all samples were frozen at -10°C until further processing. Thawed samples were run on Dowex 1 (Cl^-) columns. Columns were eluted with 2 ml dH_2O followed by 2 ml 0.01 M HCl-1M NaCl. Water and acid eluates were collected in separate scintillation vials and mixed with 15 ml of Scinti-Verse (Fisher Scientific) for counting in a Pakard Tri-Carb 300 counter (Canberra Company, Laguna Hills, CA).

RESULTS

2',5'-OAS activity in bovine PBML was monitored beginning six days before and 24 h after treatment with 3.6 x 10^6 U/kg of rBoIFN-αI1 (Fig. 1). Baseline 2',5'-OAS activity of 320 ± 131 pmols/h/10^6 cells in PBML from the untreated control calf remained constant for the duration of the experiment. Enzyme activity was 460 ± 134 pmols/h/10^6 cells in the treatment group (n = 3) prior to IFN treatment. 2',5'-OAS activity of PBML increased to 2133 ± 234 pmols/h/10^6 cells on day 1 (24 h post-treatment) in animals receiving IFN. BAM collected on days $^-$6 and 1 produced 2',5'-OAS activity similar in response to IFN as the PBML.

BAMs were collected twice at a 6 day interval due to a report of adverse affects on the composition of the BAM population or cell activities when shorter intervals are used (1). Incorporation of [8-^{14}C) adenylate into 2',5'-OAS by CE of BAM from untreated animals (n = 5) was 3180 ± 926 pmols/h/10^6 cells. Corresponding activity 2',5'-OAS by CE of BAM from treated animals (n = 3) was 5060 ± 141 pmols/h/10^6 cells ($P < 0.05$) (Fig. 2).

DISCUSSION

Interferon has been shown to inhibit many bovine viruses (2,7-9, 11). In addition to its antiviral effects in the bovine system, IFN has other effects on bovine cells including enhanced bacterial phagocytosis and antibody dependent cell-mediated cytotoxicity (ADCC) in macrophages, blood monocytes, and polymorphonuclear cells (3). IFN is currently being considered for use as a prophylactic agent for BRD. Recent studies of the 2',5'-OAS system in cattle (21,22,23) suggests its potential use as a marker in the bovine for IFN action as demonstrated in humans (20) and in murine species (10). Short and Fulton (21) found increased 2',5'-OAS levels in PBML of cattle inoculated with modified live virus vaccine and cattle treated with IFN. The study not only supports the use of 2',5'-OAS as a marker for IFN action in the bovine system, but also suggests the possibility of monitoring virus infection.

Since BAM are important in defense against microorganisms invading the lungs (3), this study measured the response of BAM from IFN treated cattle by measuring 2',5'-OAS activity in these cells. PBML from the same animals were also collected at the same time and at points in between the BAM collections. 2',5'-OAS levels were also measured in the

PBML. 2',5'-OAS activity increased in BAM of calves treated im with rBoIFN-αI_1 as compared with levels in BAM collected 6 days prior to IFN treatment from the same calves. 2'5'-OAS levels also increased in PBML of these same calves used for BAM source. These results are similar to those of Short and Fulton as described above (21). 2'5'-OAS activity in both PBML and BAM increased following IFN injection; however, basal activities varied between these two cell types with BAM having the greater activity. Variation in basal activity of 2',5'-OAS in different cell types has also been reported in studies of human peripheral white blood cells (20).

The results of this study suggest that BAM of calves treated with IFN do respond by increasing 2',5'-OAS activity. Whether or not the increased 2',5'-OAS activity is involved in the mechanism of IFN action responsible for the antiviral state or other functional alterations of IFN treated BAM remains to be determined.

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Fig. 1. Effect of intramuscular injection of IFN on PBML 2',5'-oligoadenylate synthetase activity. CE was prepared from PBML of an untreated control calf (●) and IFN treated cattle (◆). The results presented are the mean ± SD n = 1 for untreated calf and n = 3 for IFN treated calves.

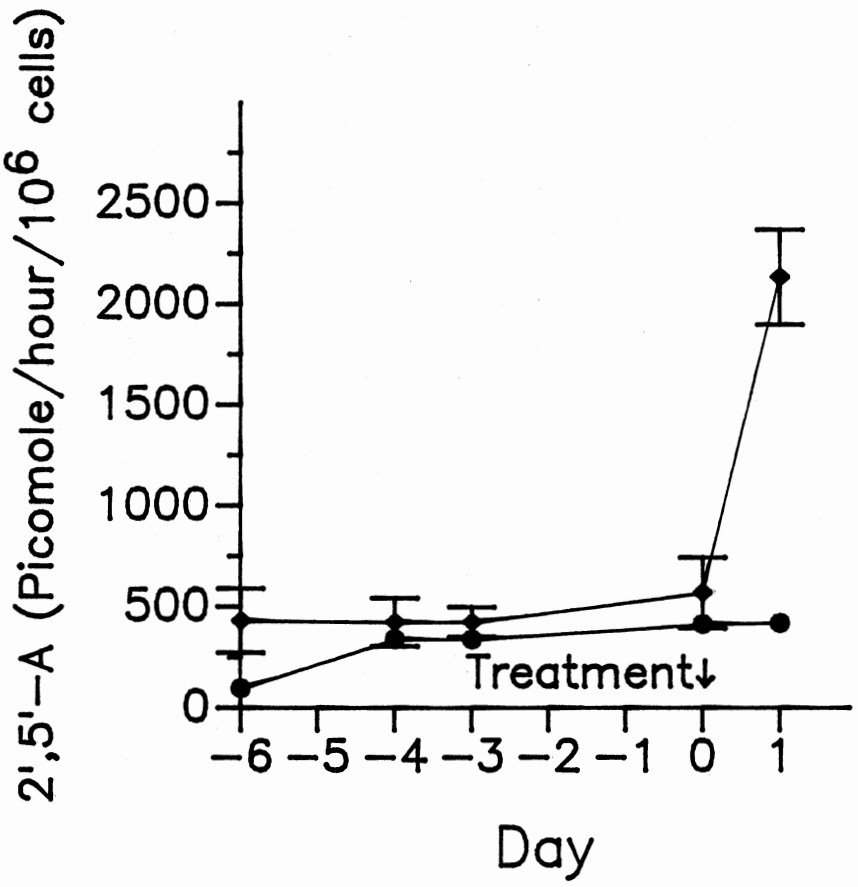
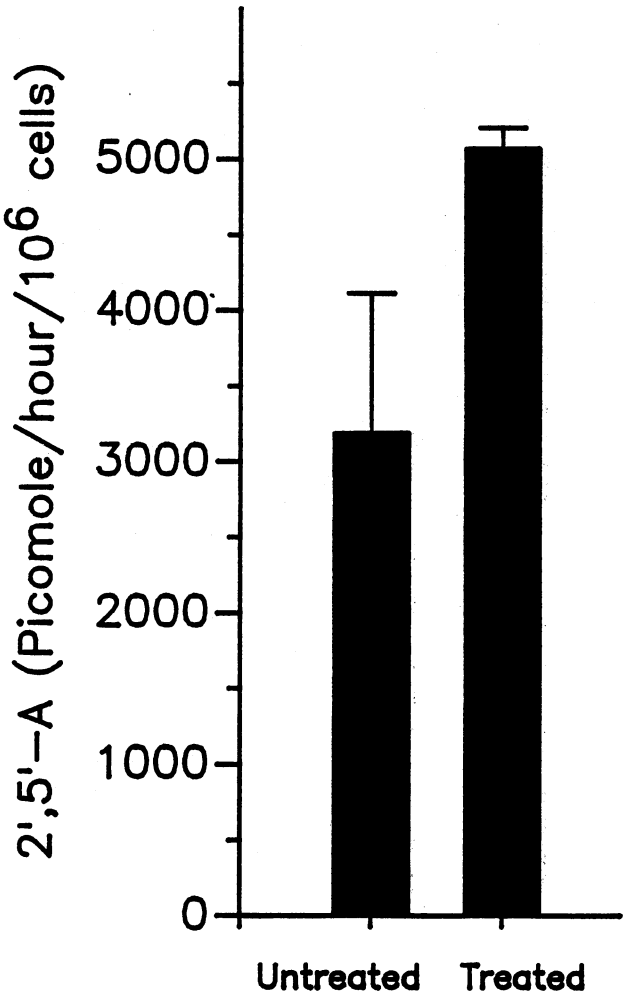

2',5'-A (Picomole/hour/10^6 cells)
2500
2000
1500
1000
500
0
Treatment↓
-6 -5 -4 -3 -2 -1 0 1
Day

Fig. 2. Effect of intramuscular injection of IFN on BAM 2',5'-oligoadenylate synthetase activity. CE was prepared from BAM of untreated control cattle and IFN treated cattle. The results presented are the mean ± SD n = 5 for untreated cattle and n = 3 for IFN treated cattle.


2',5'-A (Picomole/hour/10^6 cells)
5000
4000
3000
2000
1000
0
Untreated
Treated

CHAPTER IV

SUMMARY AND CONCLUSIONS

The purpose of this study was to measure the biological response of bovine alveolar macrophages (BAM) to recombinant DNA-derived bovine interferon alpha-I1 (rBoIFN-αI1). The *in vitro* response was monitored by two parameters: 1) yield reduction of PI-3V and 2) 2',5'-oligoadenylate synthetase (2',5'-OAS) activity in IFN treated and untreated BAM cultures. To confirm our *in vitro* findings by *in vivo* studies, we monitored 2',5'-OAS activity in BAM of calves treated with rBoIFN-αI1.

Viral yields in PI-3V challenged, IFN treated BAM were significantly ($P < 0.001$) reduced as compared to untreated, PI-3V challenged BAM. The average percent viral yield reduction was 84% and the standard deviation was ± 17% ($n = 9$). 2',5'-OAS activity in IFN treated BAM increased 80% ± 39% at the 8 h time point and 174% ± 89% at the 32 h time point compared to 16% ± 27% and 35% ± 31% at the respective time points in untreated BAM. BAM of individual animals showed varying increases in 2',5'-OAS activity. There seems to be a temporal relation between increased 2',5'-OAS activity and viral yield reduction *in vitro* in BAM. Calves which received 3.6×10^6 U/kg of rBoIFN-αI1 showed a significant ($P < 0.05$) elevation of 2',5'-OAS activity in their BAM compared to the enzyme activity in their BAM collected prior to IFN administration.

BAM isolated from all three animals in the *in vitro* study had increased 2',5'-OAS activity and decreased viral yields following treat-

ment with IFN. Although the response pattern was similar for all three calves, the magnitude of the response varied. 2',5'-OAS activity also varied in the in vivo experiment, especially in BAM collected prior to IFN injection. The variation in BAM enzyme activity of untreated calves in the in vivo experiment suggest more studies should be conducted to better establish the in vivo baseline 2',5'-OAS activity in BAM. Any future studies should use larger sample populations for a better estimate of baseline activity. Varied baseline levels and responses of 2',5'-OAS activity have also been reported in studies on humans (3,4).

The results of this study are consistent with those reported for the murine and human systems. Previous studies have indicated decreased susceptibility of BAM due to the effects of IFN (1,2,6). Wang and Wu (7) reported IFN induced de novo synthesis of 2',5'-oligoadenylate (2',5'-A) dependent endoribonuclease in cultured guinea pig macrophages. 2',5'-A dependent endoribonuclease is an RNase which degrades mRNA and rRNA, thus inhibiting virus replication. Gresser et al. (2) reported correlation between levels of 2',5'-OAS activity in mouse peritoneal macrophages and the permissiveness of these cells for vesicular stomatitis virus. In vivo induction of 2',5'-OAS activity in bovine peripheral blood mononuclear leukocytes (PBML) have been reported in response to exogenous treatment with rBoIFN-αI1 and in response to modified live virus vaccine (5). However, there are no reports on the biological effects of IFN on BAM, as related to the 2',5'-OAS system.

Induction of 2',5'-OAS activity in recombinant IFN treated BAM supports the use of 2',5'-OAS as a marker for IFN induction. The temporal relationship between viral yield reduction and increased 2',5'-OAS activity in IFN treated BAM suggests involvement of 2',5'-OAS in the

action of IFN induction of the antiviral state observed in these cells. The above findings will be beneficial in future studies on the mechanism by which IFN increases antiviral properties in BAM and monitoring responses to IFN in bovine respiratory disease (BRD) trials. The ability of individual calves to initiate a 2',5'-OAS response may be recognized in future experiments as important in their ability to withstand infections as in BRD.

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VITA

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