

Molecular aspects of persistent bovine viral diarrhea
virus infection on bovine neutrophils

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Gayle Blair Brown

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PREFACE

This is an alternate format thesis. The thesis begins with a general introduction which is followed by a review to be submitted for publication. Following the review are two separate manuscripts also to be submitted for publication. A general summary and discussion of this research concludes the thesis. Gayle B. Brown, the master's candidate, is the first author and principal investigator of the research presented.

GENERAL INTRODUCTION

The purpose of this research was to extend previous work on immunosuppression by bovine viral diarrhoea (BVD) virus and to examine the effect of in vitro cytokines on neutrophils and lymphocytes from infected cattle.

Previous work on immunosuppression and immunomodulation of bovine neutrophils is reviewed in the first section. This review was written to be useful for practicing veterinarians.

Persistent BVD infection was previously shown to have decreased neutrophil and lymphocyte function (10). To further evaluate the suppression of the neutrophil function additional, more sensitive, assays were needed. Sensitive kinetic fluorometric assays to measure the oxidative burst, elastase release and cytoplasmic calcium flux were adapted for bovine neutrophils based on human neutrophil literature (5,6,11). Some initial responses of bovine neutrophils in these assays differed from reported results of human neutrophils. In light of these differences, a comparison of bovine and human neutrophil responses to various neutrophil stimulants was made using these assays. Some interesting similarities and differences were observed. These data are presented in the second section.

After this verification and finding a single stimulant suitable for all three assays, these assays along with the traditional neutrophil assays were used to compare the function of neutrophils from cattle persistently infected with BVD virus to those from healthy uninfected

cattle and to evaluate the in vitro effects of recombinant bovine interferon gamma on neutrophil function. In addition, the in vitro effect of recombinant bovine interleukin 2 on lymphocyte blastogenesis was evaluated. These data are presented in the third section of this thesis.

All three sections will be submitted for publication.

SECTION I.

SUPPRESSION AND ENHANCEMENT OF NEUTROPHIL FUNCTION
IN INFECTIOUS DISEASES OF CATTLE

INTRODUCTION

Neutrophils are white blood cells whose primary function is to phagocytize and kill microorganisms. The importance of neutrophils in controlling infection is demonstrated by congenital defects that result in severe neutropenia or neutrophil dysfunction. These conditions are often fatal due to recurrent and/or overwhelming bacterial infections (70). More subtle conditions have been described in which neutrophil function is somewhat suppressed, with relatively normal cell counts. These subtle defects in neutrophil function predispose the animal to clinical disease. Suppression of neutrophil function has been associated with bovine respiratory disease (BRD) (8,25,55,57), bovine mastitis (38), and young calves (31,77,92). A better understanding of the neutrophil suppression associated with these conditions may lead to ways of modulating the immune system to enhance neutrophil function.

This manuscript reviews basic neutrophil physiology, discusses the neutrophil suppression associated with some infectious diseases of cattle, and reviews the current literature on biological response modifiers (BRMs) used for enhancement of neutrophil function and their potential clinical application. The immune system is complex and many components interact to bring about resistance to infection. This manuscript focuses on the neutrophil, but it must be kept in mind that suppression or enhancement of other aspects of the immune system are also occurring and are of great importance.

BASIC PHYSIOLOGY OF THE BOVINE NEUTROPHIL

Neutrophils are produced, stored and released by the bone marrow. The bone marrow readily responds to a need for neutrophils with increased production and release; neutrophilia is observed within hours of a severe bacterial infection. Once released from the bone marrow neutrophils only circulate a few hours before leaving the blood stream. In normal tissues they will survive for 1-2 days but at sites of inflammation survival is much shorter (42).

Neutrophils near the area of inflammation adhere to damaged endothelium and chemotactic factors signal neutrophils to the site of inflammation. These chemotactic factors are generated during an inflammatory process and include products of the complement cascade and arachidonic acid metabolism, bacterial products, and platelet activating factor. The neutrophils squeeze between endothelial cells and follow the chemotactic gradient to arrive at the appropriate site (22).

In connective tissue, neutrophils follow the chemotactic gradient until they come in contact with microorganisms. Contact is a necessary step for ingestion, and chemotactic factors and opsonins are largely responsible for its occurrence. Opsonins are molecules that bind to microorganisms and facilitate attachment of neutrophils with the microorganisms. Two very important opsonins are antibody (IgG) and C3b, a product of the complement cascade. Neutrophils have receptors for the Fc portion of IgG antibody and for C3b (22,86).

Upon attachment, neutrophils ingest the microorganisms by surrounding them with pseudopods from their membrane and incorporating them into a membrane bound vacuole called a phagosome (22,82).

The cytoplasm of neutrophils contains numerous granules (also called lysosomes). Bovine neutrophils contain three basic granule types defined by their staining characteristics, timing of their appearance during maturation, and their content. Myeloperoxidase, elastase and other hydrolytic enzymes are found in the primary granules. The secondary granules contain proteins including lactoferrin (an iron binding protein), collagenase, and vitamin B₁₂ binding protein (32). The tertiary granules of bovine neutrophils are the most numerous and contain cationic proteins. Cationic proteins, sometimes called defensins, disrupt the outer membrane of many microorganisms causing permeability changes and cytolysis (26-28,85).

Cytoplasmic granules move toward the phagosome upon its formation. The process of degranulation occurs when the granule contacts the phagosome and their membranes fuse forming a phagolysosome. The granule contents, now in contact with the microorganism, begin to digest and destroy susceptible organisms (22,32).

The oxidative burst is another potent killing mechanism of the neutrophil. During the process of phagocytosis, oxygen is reduced by an enzyme complex located in the cell membrane and in the membrane of the phagolysosome. Oxygen metabolites such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical (OH^-), and singlet oxygen (1O_2) are produced (22,32).

A microbicidal system that requires both degranulation and the respiratory burst is the myeloperoxidase (MPO) - H_2O_2 - halide system. Myeloperoxidase is a granule enzyme that reacts with H_2O_2 and a halide resulting in the formation of very potent bactericidal compounds (i.e., hypochlorous acid and aldehydes) and the iodination of proteins. This system is considered the most potent killing mechanism of the neutrophil (22,40).

Antibody-dependent cell-mediated cytotoxicity (ADCC) is used by neutrophils to kill virus infected cells. The neutrophil recognizes the Fc portion of the antibody bound to the viral antigen on the cell surface. The neutrophils do not ingest the virus infected cell; rather they release their granule contents and oxygen metabolites extracellularly in an attempt to kill the cell (17). Neutrophils also contribute to cellular cytotoxicity independent of antibody. The mechanism of recognition by the neutrophil for antibody independent cell-mediated cytotoxicity (AINC) is not clear (5,29).

The functions (chemotaxis, phagocytosis, degranulation, oxidative burst, ADCC, AINC) associated with normal neutrophil physiology can be measured in the laboratory (60,81). By measuring these functions it is possible to characterize the defects or the suppression of neutrophil function that occurs in infectious diseases.

BACTERIAL RESISTANCE TO NEUTROPHIL PHAGOCYTOSIS AND KILLING

Many pathogenic microorganisms have developed mechanisms to survive killing by phagocytic cells (21,78,79).

Production of toxins is one mechanism used to avoid phagocytic killing. Bacterial toxins may be cytolytic to neutrophils or inhibitory to important functions. Staphylococcus aureus produces a toxin called leukocidin which alters neutrophil function by its effect on neutrophil membranes. Streptolysin O produced by Streptococcus pyogenes inserts into neutrophil membranes and makes them permeable to ions and water. The gram negative bacteria Pasteurella haemolytica secretes leukotoxin; this toxin is inhibitory to ruminant leukocytes at low concentrations and kills them at high concentrations (4,12,13,75). Cholera toxin and pertussis toxin inhibit phagocytic cell locomotion and chemotaxis through adenylate cyclase activity (45).

Certain surface components on bacteria have antiphagocytic properties. The capsule, usually composed of polysaccharide or protein, can have many antiphagocytic characteristics (19). Capsules may inhibit the binding of opsonins or make bacterial surface binding sites for neutrophils inaccessible. Many bacterial capsules are hydrophilic, and therefore antiphagocytic, since neutrophils only phagocytize particles more hydrophobic than themselves (87,88,90).

Another strategy utilized by bacteria is camouflage. These bacteria have surface determinants that are not recognized as foreign. For example, protein A on Staphylococcus will bind the Fc portion of IgG;

therefore, the bacteria coat themselves with antibody exposing only Fab fragments (39).

Mycoplasma and gonococci inconspicuously bind to neutrophil surfaces without being ingested. These microorganisms are actually able to proliferate on the neutrophil surface (83). *Actinomyces* avoids phagocytosis by aggregating in large clumps making ingestion difficult (74).

Certain bacteria are intracellular parasites of the neutrophils and have evolved mechanisms which allow successful proliferation within the neutrophil. *B. abortus* and *H. somnus*, for example, inhibit phagosome-lysosome fusion by the release of RNA material. RNA is known to inhibit phagosome-lysosome fusion (10,11,14,34).

Bacteria which produce catalase and/or superoxide dismutase interfere with the neutrophils oxygen dependent killing by neutralizing the oxygen radicals (23,24,43).

NEUTROPHIL SUPPRESSION ASSOCIATED WITH INFECTIOUS DISEASES OF CATTLE

Bovine Respiratory Disease

In normal bovine lungs, alveolar macrophages are the predominant cell type and are responsible for the removal of foreign material and low numbers of potential pathogens which may enter the alveolus (30). In instances where high numbers of pathogens enter the lung, or there is an increase in numbers due to ineffective removal by alveolar macrophages, neutrophils respond rapidly in large numbers to the chemotactic factors resulting from the inflammatory response (89). Neutrophils enter the lung prepared to phagocytize and destroy the organisms.

In conditions where neutrophil function is suppressed, responding neutrophils are unable to control invading organism. There are at least three major factors, stress, viral infection and bacterial virulence factors which contribute to the suppression of neutrophil function and predispose to severe bacterial pneumonia (55). Usually, a combination of these factors are present in clinical bovine respiratory disease (BRD).

Common stressors in cattle production include weaning, dehorning, castration, mixing of animals and shipping. These stressors have long been associated with an increase in BRD and increased blood cortisol levels (55,57). Neutrophils from animals with elevated blood cortisol often exhibit a neutrophilia. This is due to the release of neutrophils from marginal pools and bone marrow as well as decreased egress of neutrophils from the circulation (56). Cortisol also increases the random

migration (in vitro) of neutrophils, indicating reduced expression of adherence proteins on the surface of the neutrophil and inhibited ability to arrive at the site of the invading organism (61). A more potent glucocorticoid, dexamethasone, also enhances random migration. In addition, neutrophils from animals treated with dexamethasone have diminished ability to ingest S. aureus, decreased oxidative burst, MPO-H₂O₂-halide function and ADCC. The exact functions that are decreased can vary depending on the dose administered, the protocol for time of administration, and the physiologic status of the animal. In each case, the defective function of these neutrophils increases the susceptibility of the animal to colonization by organisms and progression of the disease processes (61).

Clinical or subclinical coccidiosis may act as a stressor by suppressing bovine neutrophils and predisposing the animal to a secondary infection. Feeding decoquinate, a coccidiostat, to cattle is associated with decreased morbidity and mortality from BRD. Neutrophils from cattle fed decoquinate have reduced random migration and enhanced oxidative and MPO-H₂O₂-halide functions. Animals naturally infected with coccidia and treated with dexamethasone for 5 days developed clinical coccidiosis, and had decreased random migration, oxidative killing and MPO-H₂O₂-halide functions. Animals under the same conditions, however, when treated with decoquinate showed no clinical coccidiosis, no decrease in oxidative metabolism and only slight decrease in MPO-H₂O₂-halide function (59).

Viral agents associated with BRD include bovine viral diarrhea virus (BVD), infectious bovine rhinotracheitis virus (IBR), parainfluenza-3 virus (PI3) and bovine respiratory syncytial virus (BRSV). All four are

known to predispose to severe bacterial pneumonia, probably at least partially because of their ability to suppress neutrophil function.

All forms of bovine viral diarrhea virus infection suppress bovine neutrophil function and other aspects of the immune system. A spontaneous bacteremia was detected in calves after experimental infection with the noncytopathic virus; suggesting defective removal of bacteria from the blood stream by neutrophils (54). Animals experimentally infected with noncytopathic BVD virus exhibited decreased neutrophil MPO-H₂O₂-halide function (67). Modified live BVD virus vaccine can also suppress bovine neutrophil functions (62). Neutrophils from cattle persistently infected with BVD virus have enhanced random migration, decreased S. aureus ingestion, oxidative metabolism, degranulation, MPO-H₂O₂-halide function, and antibody-independent cell-mediated cytotoxicity. A basic defect of these neutrophils is a deficient cytoplasmic calcium flux (9). Within 5-10 seconds of a neutrophil contacting an opsonized particle, calcium is released into the cytoplasm from intracellular stores. This is an essential signal for the neutrophil to perform certain activities (48).

Infectious bovine rhinotracheitis virus infection also effects the function of bovine neutrophils. Neutrophils from animals experimentally infected with IBR have increased random migration, decreased chemotaxis, and decreased ability to phagocytize S. aureus (8). Parainfluenza-3 virus infection causes neutrophils to have decreased oxidative burst and MPO-H₂O₂-halide function (8). The role that BRSV plays in neutrophil function has not been determined, but animals infected with BRSV are at increased risk of bacterial infection (84).

The exact mechanism of viral induced neutrophil suppression is not known. It is unlikely the effect is direct, as viruses rarely infect neutrophils. The suppression may result from soluble factors produced in response to, or under the direction of, the viral infection. The neutrophil suppression predisposes the animal to bacterial infection (1).

The common bacteria associated with bovine respiratory disease are Pasteurella haemolytica, Pasteurella multocida, and Hemophilus somnus, Mycoplasma dispar and Mycoplasma bovis. Each of these microorganisms have evolved virulence factors which enable them to resist destruction by neutrophils.

Neutrophils are able to ingest and kill P. haemolytica; however, P. haemolytica produces a leukotoxin which, at low concentrations, is inhibitory to neutrophils and at high concentrations kills them. The toxin is produced by the bacteria during the log phase of growth (12,37,75,76).

Pasteurella multocida has a protective capsule of hyaluronic acid. In the presence of whole bacteria neutrophils have inhibited phagocytosis and MPO-H₂O₂-halide function. Without the capsule these inhibitory effects are not observed (71).

H. somnus is an intracellular pathogen which is able to survive within neutrophils of infected animals. H. somnus suppresses neutrophil function by inhibiting the oxidative burst (20). H. somnus also inhibits phagosome-lysosome fusion apparently because of RNA components on the surface of the bacteria (14,34).

Mycoplasma may also play a role in BRD. Mycoplasma adhere to the surface of the neutrophil. Contact with some species of mycoplasma

inhibits the neutrophil's ability to ingest a second bacteria (33). Therefore, in large numbers, mycoplasma may suppress neutrophil function and contribute to clinical disease (57).

In summary, many factors are involved in BRD, each suppressing neutrophils, as well as other defense mechanisms, to some extent. Appropriate combinations of suppressive factors leave the animal susceptible to severe disease.

Bovine Mastitis/Metritis

The primary line of defense against invading organisms in the bovine mammary gland is the teat canal. The teat canal is lined with keratin which has bactericidal activity and provides a barrier to invading pathogens (51).

The neutrophil is an important second line of defense and is the predominant cell type (comprising 80 - 90% of the somatic cells) in the inflamed udder. The somatic cell count in subclinical mastitis averages about 700,000 cells/milliliter (ml) of milk, while clinical mastitis cell counts may be as high as 2 million cells/ml of milk (50).

Milk neutrophils have decreased phagocytic and bactericidal activity when compared to circulating neutrophils. Neutrophils deplete their energy stores by ingesting fat globules and casein in the milk. In addition, the prominent IgG subtype in bovine milk, IgG1, is not a good opsonin (IgG2 is important) (46). Other opsonins, i.e., complement fragments, are not found in high concentration in milk. Low oxygen tension in the udder affects the pH of the phagolysosomes and may also

contribute to the decreased bactericidal activity of milk neutrophils (44). Despite the inhibited function in the milk, neutrophils are very important for mammary gland disease resistance. This is demonstrated by the use of intramammary devices which increase neutrophil numbers in the teat and enhance resistance to environmental pathogens (47,49).

Approximately 50% of clinical bovine mastitis occurs during the first three months post calving, with new infections typically occurring the last two weeks of the dry period (51). A correlation between the incidence of mastitis and decreased function of circulating neutrophil and lymphocyte has been observed. The major suppression of neutrophil function occurs one week postpartum. In vitro assays showed that oxidative metabolism and MPO-H₂O₂-halide function were significantly suppressed (38). Possible factors contributing to this suppression include stress of parturition and lactation, altered hormonal levels, and negative energy balance postpartum.

Neutrophils are important in protection of the uterus from bacterial colonization. During the estrous cycle serum progesterone and estrogen concentrations fluctuate, low serum progesterone and high serum estrogen followed by high serum progesterone and low serum estrogen, etc. There is increased incidence of metritis when serum progesterone is high, and when serum estrogen is high there is resistance of the uterus to infection. Evaluation of neutrophil function during the estrous cycle revealed high serum progesterone was associated with inhibited oxidative metabolism and MPO-H₂O₂-halide function and enhanced random migration and ADCC. High serum estrogen concentration was associated with enhanced random migration and increased serum cortisol levels. The enhanced random migration by

neutrophils may be due to the effects of cortisol rather than estrogen (66). Another experiment examined neutrophil function in steers treated with high doses of estradiol or progesterone. In steers treated with estradiol no significant decrease in neutrophil function was observed. However, steers treated with progesterone had increased random migration and decreased MPO-H₂O₂-halide function (68). In summary, these data indicate hormonal changes, as well as other factors, in cows during the estrous cycle, pregnancy and parturition alter neutrophil function, and decreased function correlates with times of increased susceptibility to infection.

Diseases of Young Calves

Young animals have increased susceptibility to infectious diseases. Calves are born immunocompetent but are immunologically naive. The means of protection from disease in these young animals include native defenses (e.g., epithelial barriers, complement and phagocytic cells) and maternal antibodies obtained through the colostrum (6).

Neutrophil function of young calves when compared to adults is decreased and may contribute to the susceptibility of calves to infectious diseases. Neutrophils from neonatal calves exhibit normal adhesive and morphologic changes in vitro when stimulated by complement fragments and ingestion of S. aureus is normal (92). Decreased neutrophil functions in neonatal calves include the MPO-H₂O₂-halide system, the oxidative burst and ADCC. Inhibited oxidative metabolism was observed in calves 4-5 weeks of age or younger, and depressed MPO-H₂O₂-halide function and ADCC was

still evident in calves 16 - 19 weeks of age (31,77). The mechanism for the decreased function has not been determined. However, one investigation determined that neutrophils from a fetus (210-220 days of gestation) did not exhibit defective oxidative metabolism. Therefore, the defect was not simply a matter of development (18).

ENHANCEMENT OF NEUTROPHIL FUNCTION

Neutrophil suppression in cattle is demonstrated in association with many of the infectious diseases of cattle. Experimental data suggests that reversal of the suppression may improve the health of cattle (2,15).

Products used for the enhancement of immune function are often called immunomodulators. There are two basic types of BRMs, exogenous and endogenous. The exogenous immunomodulators include bacteria or bacterial derived products (e.g., *Bacillus Calmette-Guerin* (BCG), endotoxin, *Propionibacterium acnes*) and synthetic chemicals (e.g., levamisole and lipoidal amines). One mechanism of action of the exogenous immunomodulators is to induce the release of endogenous immunomodulators. The endogenous immunomodulators include proteins that are produced and secreted by cells (cytokines). Some examples of these proteins include interferon (IFN), tumor necrosis factor (TNF) and granulocyte macrophage-colony stimulating factor (GM-CSF) (58,80).

Conditions which are candidates for the use of immunomodulators are those where immunosuppression is a key component of the pathogenesis of the disease process, and the occurrence of the immunosuppression is predictable; immunomodulators have their greatest effect if used prior to the development of clinical signs, or early in the disease process. The conditions of cattle that were just discussed are all candidates for the use of immunomodulators.

Several of the immunomodulators have been examined for their enhancement of bovine neutrophil function. The approach used to examine

immunomodulators for use in cattle varies; one approach is to determine the molecular and cellular aspects of the immunosuppression, examine the in vitro and in vivo effect of various dosages of the immunomodulator on neutrophil function from healthy and immunosuppressed cattle, then design challenge studies to determine the in vivo effect of immunomodulators on the disease process.

The exact mechanism of action of the immunomodulators is not known. In general, cytokines bind to receptors on neutrophils resulting in activation of the neutrophil and enhancement of various functions (80).

Avidine, a lipoidal amine, is known to be an interferon inducer. Avidine, when administered to calves, enhanced the bactericidal activity of neutrophils (91). In healthy steers, avidine enhanced phagocytosis and cytotoxic functions of neutrophils. At high doses avidine inhibited the MPO-H₂O₂-halide function of neutrophils from these steers. Avidine was also examined for its effect in cattle immunosuppressed with dexamethasone. Avidine reversed most of the neutrophil suppression induced by the dexamethasone treatment (64). Since avidine induces the animal to produce interferon and probably numerous other cytokines, it was decided to evaluate the influence of cytokines in vitro.

Supernatant from bovine mononuclear cells stimulated with antigen was collected. This antigen-induced lymphokine preparation was incubated with bovine neutrophils in vitro. Many of the same functions which were enhanced in cattle treated with avidine were enhanced following incubation with the lymphokine preparation (41). The active component of the lymphokine preparation was not known, but it was known to contain interferon.

Interferon gamma, produced by T lymphocytes in response to antigen stimulation, has many modulatory activities on neutrophils. DNA technology has made recombinant cytokine products available. Therefore, the next step was to examine the effect of recombinant bovine IFN gamma (rboIFN gamma) on neutrophils from normal cattle and from cattle immunosuppressed with dexamethasone. These experiments demonstrated enhancement of neutrophil functions similar to avridine and lymphokine (81).

Following the positive results obtained with in vitro and in vivo rboIFN gamma, a challenge study in calves was performed to examine the effect of rboIFN gamma on experimental infection with H. somnus. Young calves were treated with dexamethasone and/or rboIFN gamma and challenged with H. somnus. This model involves at least three conditions for depressed neutrophil function: dexamethasone, H. somnus virulence factors, and young animals. Calves immunosuppressed with dexamethasone and administered rboIFN gamma had significantly less fibrinous pneumonia after H. somnus challenge than those calves administered dexamethasone but not rboIFN gamma (15). This indicates that an immunomodulator which is capable of enhancing phagocytic cell function can have a significant beneficial effect in immunosuppressed calves with bacterial pneumonia.

Interferon alpha, produced by leukocytes in response to virus infection and other stimuli, has antiviral activity but has also shown some indication of enhancing the function of neutrophils. In vitro incubation of bovine neutrophils with rboIFN alpha has some activating effect on the neutrophils. Random migration was inhibited, phagocytosis was enhanced and there was no effect on chemotaxis or oxidative burst

except at high concentrations where oxidative metabolism was inhibited (7,73). Examination of neutrophil function from calves following in vivo administration with IFN alpha is variable. There is a tendency for inhibited migration and enhancement of oxidative metabolism (7). A challenge study was performed to examine the potential for IFN alpha in controlling an infectious disease. In this challenge system the calves were immunosuppressed by exposure to IBR virus. Following the immunosuppression the calves were exposed to P. haemolytica. Calves treated with IFN alpha, in vivo, before exposure to IBR virus endured the P. haemolytica challenge (2,3) better than calves not treated with IFN alpha.

Other immunomodulators have been examined for ability to enhance bovine neutrophil function. Several of these show promise, but they have apparently not yet been used in infectious disease models.

Levamisole, a drug most commonly used as an anthelmintic, enhances certain immune function in vitro of human cells (52). In cattle, levamisole enhanced the chemotactic response of neutrophils following in vitro incubation and also 90 minutes after in vivo administration (35). However, levamisole, administered in vivo to cattle immunosuppressed with dexamethasone, had no effect on neutrophils function (63). Similarly, thiabendazole was without effect on bovine neutrophil function (36,69).

Nutrition is recognized to influence the immune function. One study examined the effects of ascorbic acid (Vitamin C) on neutrophil function. Ascorbic acid enhanced the oxidative burst and ADCC of neutrophils from healthy cattle. In addition, administration of ascorbic acid to cattle immunosuppressed with dexamethasone reversed the suppression of the

neutrophil functions of random migration, oxidative metabolism and ADCC (65).

Tumor necrosis factor (TNF) secreted by macrophages and monocytes during an immune response acts on neutrophils. In vitro incubation of bovine neutrophils with recombinant human (rhu) TNF increased the oxidative burst compared to controls (72). In vitro incubation of bovine neutrophils with recombinant bovine (rbo) TNF alpha inhibited migration (directed and random), and enhanced AINC. No enhancement of oxidative metabolism was detected in this investigation (16). However, many of the conditions in these two investigations were different (e.g., rhuTNF vs rboTNF).

Granulocyte macrophage - colony stimulating factor (GM-CSF) is a cytokine released from T cells stimulated by an antigen. In vitro incubation of neutrophils from healthy cattle with rboGM-CSF enhanced chemiluminescence, phagocytosis and cytotoxic cell functions. These neutrophil functions in cattle immunosuppressed with dexamethasone were enhanced by incubation with rboGM-CSF also. The enhancement of neutrophil function from dexamethasone treated animals was greater than the enhancement of controls (53).

It is apparent that neutrophils with suppressed function can be activated to have improved function by administration of immunomodulators, and in general, the immunomodulators have more modulating activity in neutrophils with suppressed function. The use of immunomodulators in cattle production will be beneficial during those predictable times of immune suppression and increased disease. However, further work in the areas of combination immunomodulator administration, timing of

administration, dosage of administration, and low cost production is needed before immunomodulators can be used effectively.

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SECTION II.

COMPARISON OF THE RESPONSE
OF BOVINE AND HUMAN NEUTROPHILS TO VARIOUS STIMULI

ABSTRACT

Elastase release, oxidant production and cytoplasmic Ca^{++} fluxes by bovine and human neutrophils were compared using sensitive kinetic assays on a photon-counting spectrofluorometer. The stimulants used were phorbol myristate acetate (PMA), cytochalasin B, zymosan opsonized with bovine complement (bOZ) or human complement (hOZ), calcium ionophore, formyl-methionyl-leucyl-phenylalanine (FMLP), and concanavalin A (Con A). The respiratory burst of bovine and human neutrophils was stimulated by PMA and OZ but not by cytochalasin B, or calcium ionophore. Concanavalin A weakly stimulated this response in human neutrophils but not bovine. Formyl-methionyl-leucyl-phenylalanine stimulated the respiratory burst of human but not bovine neutrophils. For evaluation of elastase release, human neutrophils were pretreated with cytochalasin B for 5 minutes and then stimulated. Cytochalasin B alone did not stimulate elastase release from human neutrophils. Phorbol myristate acetate, calcium ionophore, hOZ, FMLP and Con A did stimulate human neutrophils pretreated with cytochalasin B to release elastase. Human OZ was also able to stimulate elastase release from human neutrophils not pretreated with cytochalasin B. Some bovine neutrophils released elastase in response to cytochalasin B alone. Those bovine neutrophils that did not release elastase in response to cytochalasin B alone released elastase when stimulated with Con A or calcium ionophore after cytochalasin B pretreatment. Bovine neutrophils did not release elastase in response to FMLP or PMA with or without cytochalasin B pretreatment, but did release elastase in response

to bOZ alone. Total elastase activity of bovine neutrophils was determined to be about fifty times less than that of human neutrophils. Intracellular calcium fluxes were stimulated in human neutrophils by calcium ionophore, FMLP, hOZ, and Con A but not by PMA or cytochalasin B. Bovine neutrophil calcium fluxes were stimulated by calcium ionophore, ConA and bOZ; cytochalasin B also stimulated bovine neutrophils to increase cytoplasmic calcium concentration. Cytoplasmic calcium fluxes were not stimulated in bovine neutrophils by PMA or FMLP. In summary, human and bovine neutrophils respond similarly to calcium ionophore and OZ, but differently to PMA, cytochalasin B, Con A and FMLP.

INTRODUCTION

Neutrophils are essential for host defense against many bacterial and fungal agents. When neutrophils are activated in response to inflammatory stimuli the major events that occur include: chemotaxis, phagocytosis, secretion of enzymes (degranulation), and the oxidative burst of metabolism (45). The ability of neutrophils to carry out these functions can be influenced by various factors. For example, cytokines may enhance neutrophil function (47); stress (24), corticosteroids (8,9,19,20,39), and viruses (1) may suppress neutrophil function.

The activation of bovine neutrophils involves a complex series of events. Investigation of the kinetics of these events at the subcellular and molecular level will help to elucidate how bovine neutrophil function is modulated by various factors.

Assays to measure the kinetics of the oxidative burst (23), degranulation (41) and intracellular calcium fluxes (18) have been developed for human neutrophils using a photon-counting spectrofluorometer. These assays have not been previously used to study bovine neutrophil function. It is reasonable to assume that these assays could be adapted for use in bovine neutrophil research, since only minor differences between bovine and human neutrophils have previously been reported. For example, human neutrophils have receptors for n-formyl-methionyl-leucyl-phenylalanine (FMLP) and bovine neutrophils do not (17). Bovine neutrophils have enhanced antibody independent cytotoxic activity when pretreated with recombinant bovine gamma interferon; this was not

observed in human neutrophils pretreated with human gamma interferon (46). Bovine neutrophils do not contain lysozyme and differ in some other granule enzyme activities as compared to human neutrophils (14,35). The purpose of this research was to compare the response of human and bovine neutrophils to various neutrophil stimulants using kinetic assays for the oxidative burst, elastase release, and intracellular calcium fluxes and to determine the suitability of these assays for bovine neutrophil research.

MATERIALS AND METHODS

Neutrophil Isolation

Bovine

Neutrophils from 12 - 24 month old healthy steers were isolated as previously described (38). Briefly, citrated peripheral blood was centrifuged, and the plasma and buffy coat discarded; the packed red blood cells were lysed with cold, distilled water. The lysis step was repeated, and the neutrophils were washed and resuspended in 0.015 M phosphate buffered saline solution, pH 7.2 (PBSS) at 5×10^7 cells/ml. Purity of the neutrophil sample is greater than 90 percent.

Human

Neutrophils from healthy adult volunteers were isolated from citrated peripheral blood as previously described (37). Briefly, the blood was centrifuged, and the plasma and buffy coat discarded; the packed red blood cells were lysed with cold ammonium chloride solution. The lysis step was repeated, and the neutrophils were washed and resuspended in Hank's balanced salt solution without phenol red (HBSS) (Gibco Lab., Grand Island, NY) at 5.0×10^7 cells/ml. Purity of the neutrophil sample is greater than 90 percent.

Neutrophil Stimulants

Each of the stimulants was purchased from Sigma Chemical Company, St. Louis, MO. Phorbol myristate acetate (PMA), cytochalasin B and calcium ionophore, A23187, were each dissolved in dimethyl sulfoxide (DMSO) to a concentration of 2.0 mg/ml and stored at -20°C as a stock solution. The final concentration for each stimulant in solution with neutrophils was: PMA, 1.0 ug/ml; cytochalasin B, 0.1, 1.0, or 10.0 ug/ml; calcium ionophore, 0.5 ug/ml. Bovine opsonized zymosan (bOZ) was prepared by incubating zymosan A with fresh bovine serum as previously described (38). Human opsonized zymosan (hOZ) was prepared by washing three grams of zymosan A in PBS, resuspending the zymosan in Earle's Balanced Salt Solution (Gibco) and incubating the suspension with fresh human serum for 30 minutes at 37°C with constant stirring. After washing in EBSS, the hOZ was resuspended in 300 ml of HBSS. Both types of OZ were used at a final concentration with neutrophils of 1.0 mg/ml. N-formyl-methionyl-leucyl-phenylalanine (FMLP) was dissolved in DMSO at a concentration of 4.4 mg/ml and then diluted in PBSS to 21.0 ug/ml and stored at -20°C as a stock solution. The FMLP was used at a final concentration in solution with neutrophils of 2.1 ug/ml. Concanavalin A (Con A) was dissolved in distilled water to a concentration of 50 mg/ml and then diluted in M199 (Gibco) to 2.5 mg/ml and stored at -70°C as a stock solution. The Con A was used at a final concentration in solution with neutrophils of 250 ug/ml. Each stimulant was prepared daily from the stock solution.

Kinetic Assays for Neutrophil Function

The kinetic assays were performed using an SIM 8000C photon-counting spectrofluorometer (SIM Instruments, Inc., Urbana IL). This instrument has three photomultiplier tubes in T format optics and a stirred thermostated sample chamber with an injection port for addition of reagents during the assay. The SIM 8000C is controlled by an IBM PC computer interfaced to a Hewlett-Packard 7470A plotter.

Oxidant production

Oxidant production was measured as previously described (23). Briefly, upon appropriate stimulation, neutrophils produce superoxide anion which, in the presence of superoxide dismutase (Sigma) is converted to hydrogen peroxide (H_2O_2). The H_2O_2 then oxidizes p-hydroxyphenylacetate (PHPA) (Sigma), in the presence of horseradish peroxidase (Sigma), to a fluorescent product, $PHPA_2$. This fluorescent product, when excited at 340 nm, emits light at 400 nm. Each cuvette contained 2.5 ml of HBSS, 5.0×10^6 neutrophils and 75 μ l of a cocktail made by combining superoxide dismutase stock solution (8.0 mg/ml PBSS), horseradish peroxidase stock solution (8.0 mg/ml PBSS), and PHPA stock solution (10.0 mg/ml PBSS) at a ratio of 10:10:25, respectively. The cuvettes were prewarmed to 39°C and then placed into the sample chamber. The appropriate stimulant was added 20 seconds after the initiation of the assay. The excitation wavelength was 340 nm and the emission wavelength was 400 nm. Light emission was integrated for 2 seconds and plotted at 2 second increments for 400 seconds. A standard curve of concentrations of

H₂O₂ was prepared by adding known amounts of H₂O₂ to the cocktail and measuring the fluorescence intensity. The results were expressed as nmoles of H₂O₂ produced per five million neutrophils. The assay conditions were the same for bovine and human neutrophils.

Elastase release

Elastase release was measured as previously described (41). Methylsuccinylalanylalanylprolylvalyl-methylcoumarin amide (peptidyl-MCA) (Peninsula Laboratories, Inc., Belmont, CA) was dissolved in DMSO and then diluted in PBSS to a concentration of 1.25 mg/ml and stored at -20°C as a stock solution. Each cuvette contained 2.5 ml of HBSS, 5.0×10^6 neutrophils and 75 ul of the peptidyl-MCA stock. The cuvette was prewarmed to 39°C and then placed in the sample chamber. The stimulants were added 20 seconds after the initiation of the assay. Upon appropriate stimulation of the neutrophil, elastase is released from neutrophil granules and cleaves the substrate, liberating the fluorophore aminomethylcoumarin (AMC) which when excited at 380 nm will emit light at 490 nm. Light emission was integrated for 2 seconds and plotted at 2 second increments for 400 seconds. The assay conditions for bovine and human neutrophils were essentially the same, except that the high voltage setting for use with bovine neutrophils was higher to compensate for the decreased elastase activity in those cells. A standard curve was made using known amounts of aminomethylcoumarin (Peninsula Laboratories, San Carlos, CA) and measuring its fluorescence intensity. The amount of fluorescent product produced by neutrophils was expressed as nmoles AMC per five million neutrophils.

Total elastase activity

Five million neutrophils were placed in a cuvette with 2.5 ml distilled water and allowed to lyse for 5 minutes. The cuvette was then placed in the sample chamber, and 20 seconds after the initiation of the assay 75 ul of peptidyl-MCA was injected into the cuvette. The rate of substrate cleavage was used to determine elastase activity. A standard elastase curve was made using various concentrations of purified porcine elastase (Sigma) with an activity of 70 units per mg protein; one unit is defined by solubilizing one mg elastin in 20 minutes. at pH 8.8 at 37°C. The standard curve was used to determine the total elastase activity per cell. The rate of substrate cleavage was determined for four human neutrophil preparations and four bovine neutrophil preparations.

Cytoplasmic calcium flux

Fura 2/acetoxymethylester (AM) (Calbiochem-Behring Diagnostics, San Diego, CA) was used to measure fluxes in intracellular calcium concentration as previously described (18). Fura 2/AM was dissolved in DMSO at 5.0 mg/ml and stored at -20°C as a stock solution. The AM allows the Fura 2 to cross the cell membrane. Once inside the cell the AM is cleaved off by intracellular hydrolases. Fura 2 shifts its peak absorbance of 380 nm to 340 nm upon binding calcium while emitting light at 510 nm. Fifty million neutrophils were loaded with Fura 2 by incubating the neutrophils with an aliquot of the stock solution (diluted in HBSS to a final concentration in solution with neutrophils of 10.0 ug/ml) for 30 minutes at 39°C. After incubation the cells were washed, and 5.0×10^6 Fura 2 loaded neutrophils were added to a cuvette containing

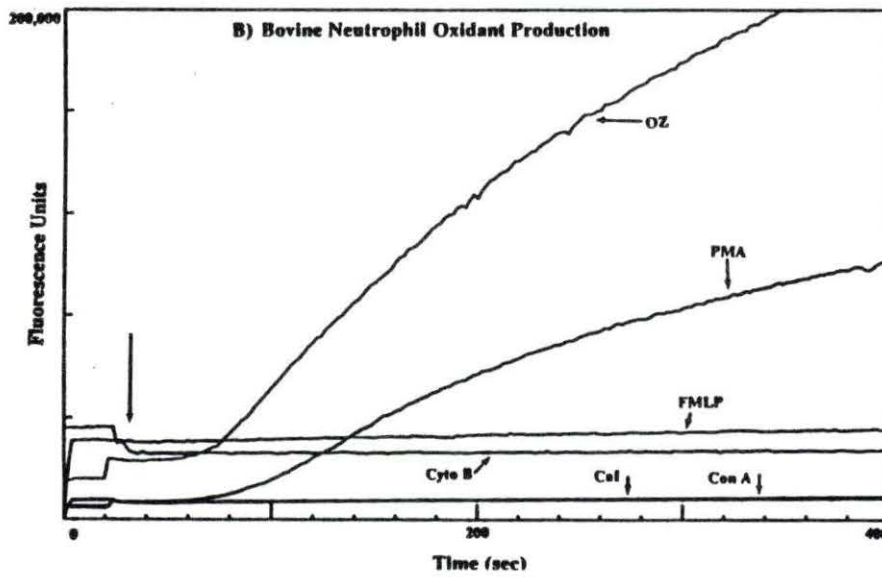
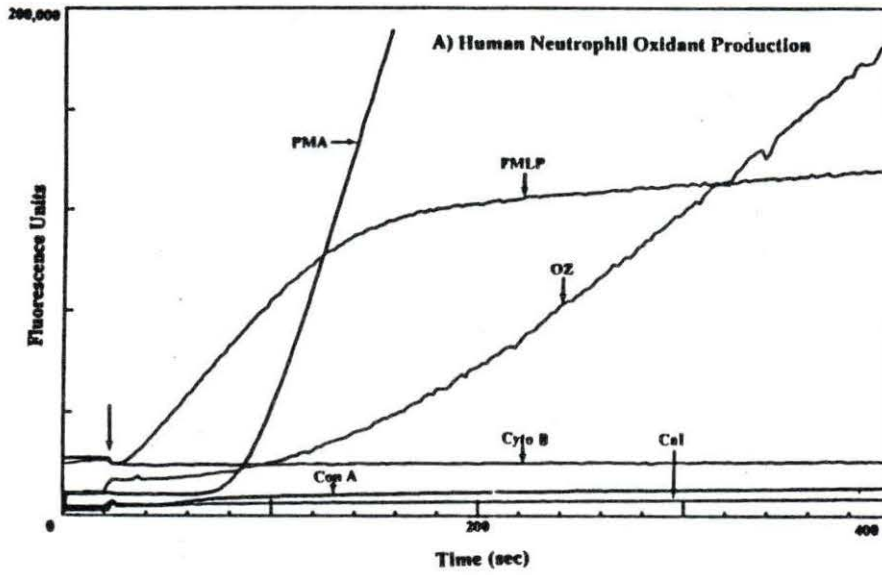
2.5 ml of HBSS. The cuvettes were prewarmed to 39°C and placed into the sample chamber. The appropriate stimulant was added 10 seconds after the initiation of the assay. Fluorescence ratios were recorded by measuring the light emission at 510 nm elicited when the excitation wavelength alternated between 340 and 380 nm. The corrected fluorescence ratios were plotted over a 100 second time period. Cytoplasmic calcium concentrations were determined for the baseline values and for the peak values using the following equation (18): $[Ca^{2+}]_i = K_d[(R - R_{min})/(R_{max} - R)](S_{f2}/S_{b2})$ where the K_d for Fura2 is 224 nM and R is the fluorescence ratio for the unknown. R_{min} is the fluorescence intensity ratio after the cells are lysed (0.1% TritonX 100), and ethylenediaminetetraacetic acid (EDTA) is added so the dye has no calcium bound; R_{max} is the fluorescence intensity ratio after the cells are lysed and calcium chloride is added so the dye is calcium saturated. S_{f2} is the fluorescent intensity at excitation of 380 nm when dye has no calcium bound; S_{b2} is the fluorescent intensity at 380 nm when dye is saturated with calcium.

RESULTS

Oxidant production

Presence or absence of stimulation of the oxidative burst for bovine and human neutrophils was similar for each of the six stimulants except Con A and FMLP (Fig. 1). When human neutrophils were stimulated with FMLP there was a rapid increase in product formation which then leveled off; bovine neutrophils did not respond to FMLP. The kinetics of oxidant production in response to PMA and OZ was different for human and bovine neutrophils. Phorbol myristate acetate was a strong stimulant of human neutrophil oxidant production. There was a short lag time and then a rapid increase in product following PMA stimulation. Stimulation of human neutrophils with hOZ caused a longer lag time than PMA and a slower rise. In bovine neutrophils stimulated with PMA there was a lag time and then a slow increase in product formation. Bovine OZ was a strong stimulant of oxidant production in bovine neutrophils with a shorter lag time and a faster rise than PMA. Concanavalin A was a weak stimulant of oxidant production in human neutrophils; there was no response by bovine neutrophils. Oxidant production was not stimulated for either species by cytochalasin B or calcium ionophore (Fig. 1).

Figure 1. Neutrophil oxidant production. A) Human B) Bovine. The data are presented as fluorescence units versus time in seconds. A change of 20,000 fluorescence units is equal to 1.0×10^{-8} moles H_2O_2 produced. This figure shows a representative sample of the results obtained for each of the stimulants tested. The stimulant was added to the cell suspension 20 seconds after the initiation of the assay (indicated by the arrow). The stimulants used were: a) bOZ or hOZ, 1.0 mg/ml b) PMA, 1.0 ug/ml c) Cytochalasin B (Cyto B), 1.0 ug/ml d) FMLP, 2.1 ug/ml e) Calcium ionophore (CaI), 0.5 ug/ml f) Con A, 250 ug/ml



Elastase release

In human neutrophils 1.0 ug/ml cytochalasin B did not cause elastase release and was used as a pretreatment for the elastase assay as previously reported (16). The human neutrophils responded strongly to calcium ionophore, hOZ, Con A, and PMA (Fig. 2). There was a short lag time and then a rapid increase in product formation. Human neutrophils responded less strongly to FMLP. There was an initial rapid increase that tapered off. Human neutrophils did release elastase in response to hOZ alone; the response was lower in magnitude than the response to hOZ of human neutrophils pretreated with cytochalasin B (Fig. 2b).

The response of bovine neutrophils to cytochalasin B varied amongst animals. Neutrophils from some animals released elastase in response to cytochalasin B (10.0, 1.0, 0.1 ug/ml) in a titratable manner. Others were stimulated to release elastase by 10.0 ug/ml cytochalasin B but not by 1.0 ug/ml, and some bovine neutrophils did not respond at all to cytochalasin B (Fig. 3). There was no stimulation of elastase release in any of the bovine neutrophil preparations to 0.01 ug/ml cytochalasin B.

In those neutrophil preparations for which cytochalasin B did not cause elastase release, Con A did cause elastase release but only after cytochalasin B pretreatment (Fig. 3b). The concentration of cytochalasin B used as pretreatment for the elastase release assay varied. For example, neutrophils that would release elastase in response to 10.0 ug/ml cytochalasin B would be treated for five minutes with ten fold less cytochalasin B (1.0 ug/ml) prior to stimulation. Neutrophils that released elastase in response to 1.0 ug/ml cytochalasin B would be pretreated for five minutes with 0.1 ug/ml cytochalasin B and then

stimulated to release elastase by a second stimulant. None of the bovine neutrophils tested released elastase in response to Con A alone. The release of elastase by bovine neutrophils in response to calcium ionophore was similar to that of Con A (Fig. 3c).

Bovine neutrophils did not release elastase in response to PMA or FMLP whether or not they were pretreated with cytochalasin B (Fig. 4). Elastase release was stimulated by bOZ alone in all animals (Fig. 4); pretreatment with cytochalasin B in those animals that did not release elastase in response to cytochalasin B directly did not affect the response to bOZ.

Total elastase activity

The mean (\pm SEM) total elastase activity of bovine neutrophils was approximately 0.102 (\pm 0.014) ug/million neutrophils; the elastase activity of human neutrophils was approximately 4.87 \pm 0.45 ug/million cells. In comparison, the bovine neutrophils had almost fifty fold less elastase activity than human neutrophils. This difference may be due to a difference in total elastase in the neutrophils, a difference in elastase activity for the substrate peptidyl-MCA, and/or the presence of elastase inhibitors.

Figure 2. Human Neutrophil Elastase Release. The data are presented as fluorescence units versus time in seconds. A change of 20,000 fluorescence units is equal to 1.0×10^{-8} moles of product (aminomethylcoumarin) formed. This figure shows a representative sample of the results obtained for each of the stimulants tested. Each sample was incubated 5 minutes with 1.0 ug/ml Cyto B before the assay (Fig. 2a) except where otherwise indicated (Fig. 2b). The stimulant was added 20 sec after the initiation of the assay (indicated by the arrow). The stimulants are the same as those defined in Figure 1

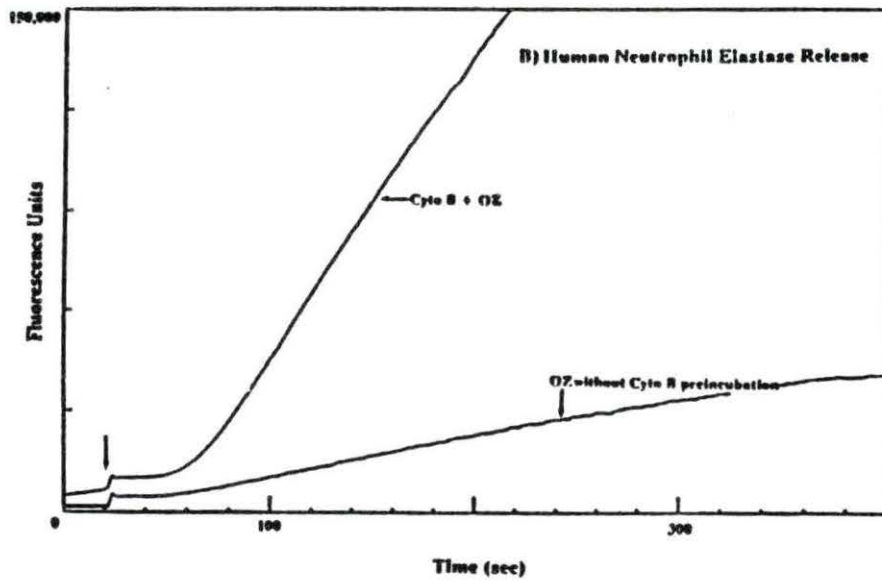
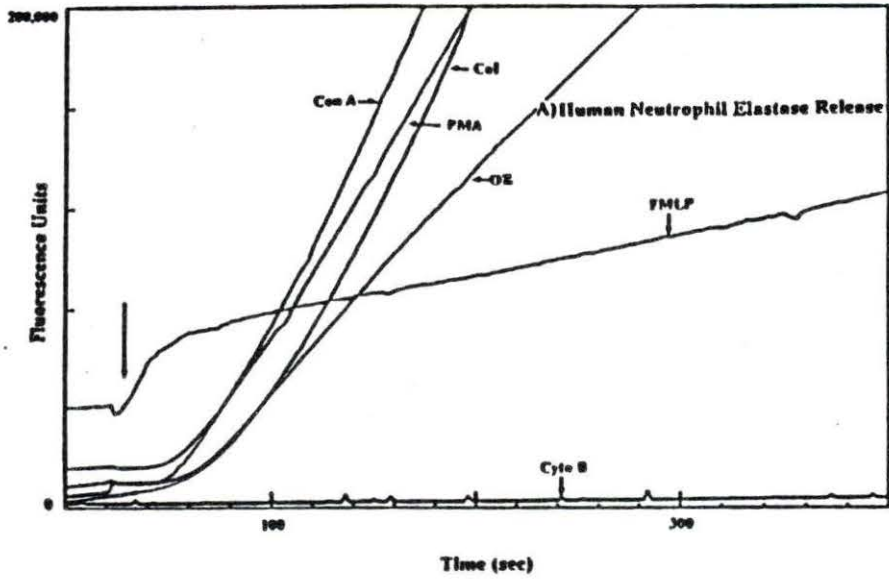
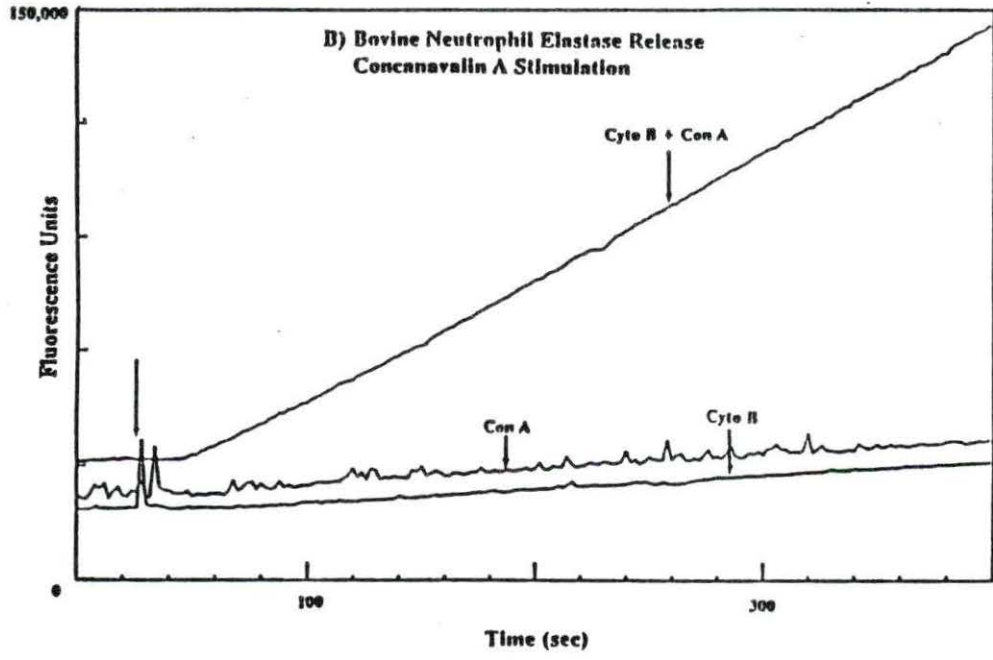
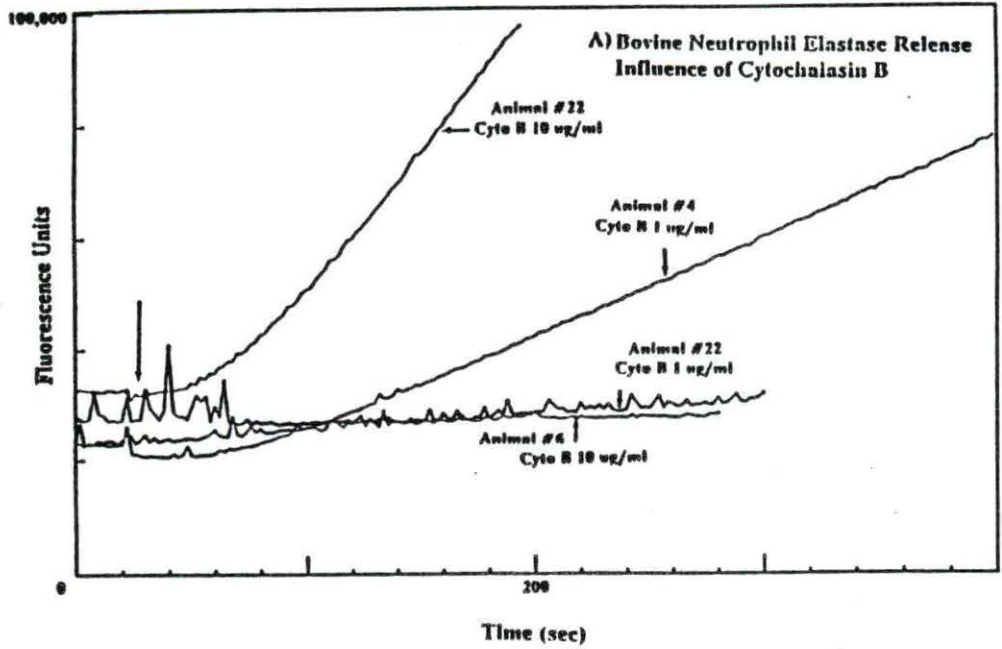


Figure 3. These graphs represent the variable response of bovine neutrophils to Cyto B, and the influence of Cyto B pretreatment on the response to Con A and CaI. The data are presented as fluorescence units versus time. A change of 20,000 fluorescence units is equal to 4.1×10^{-11} moles of product (aminomethylcoumarin) formed. The stimulants were added 20 seconds after the initiation of the assay (indicated by the arrow). A) Neutrophil elastase release in response to Cyto B alone, added at 20 seconds without preincubation. B) Neutrophil elastase release in response to Cyto B alone (1.0 ug/ml), Con A alone (250 ug/ml), or Con A (250 ug/ml) after 5 minutes preincubation with Cyto B (1.0 ug/ml). C) Neutrophil elastase release in response to Cyto B alone (1.0 ug/ml), CaI alone (0.5 ug/ml), or CaI (0.5 ug/ml) after 5 minutes preincubation with Cyto B (1.0 ug/ml)



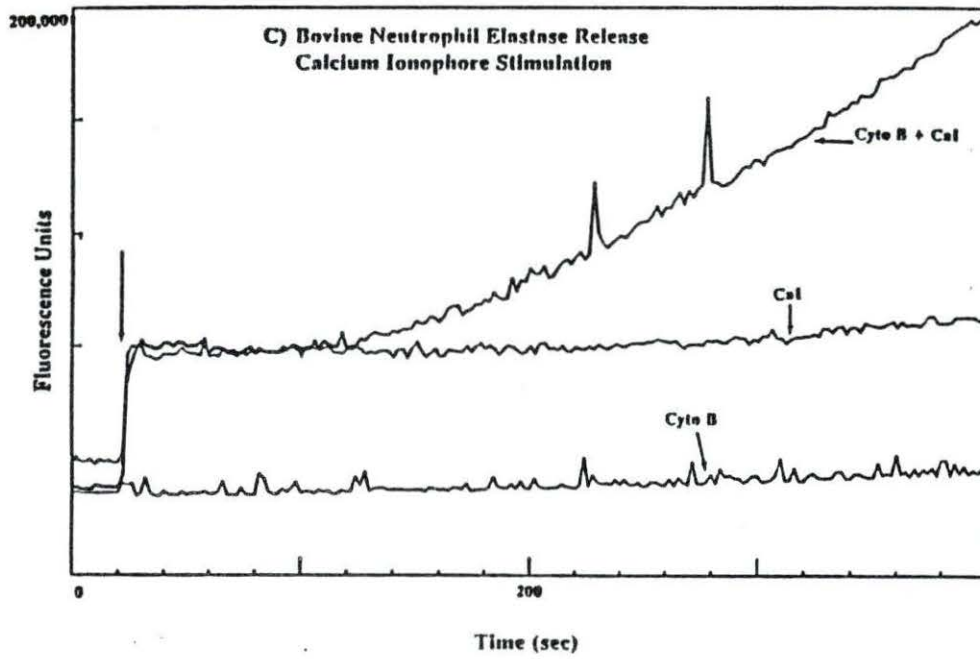


Figure 3 (continued)

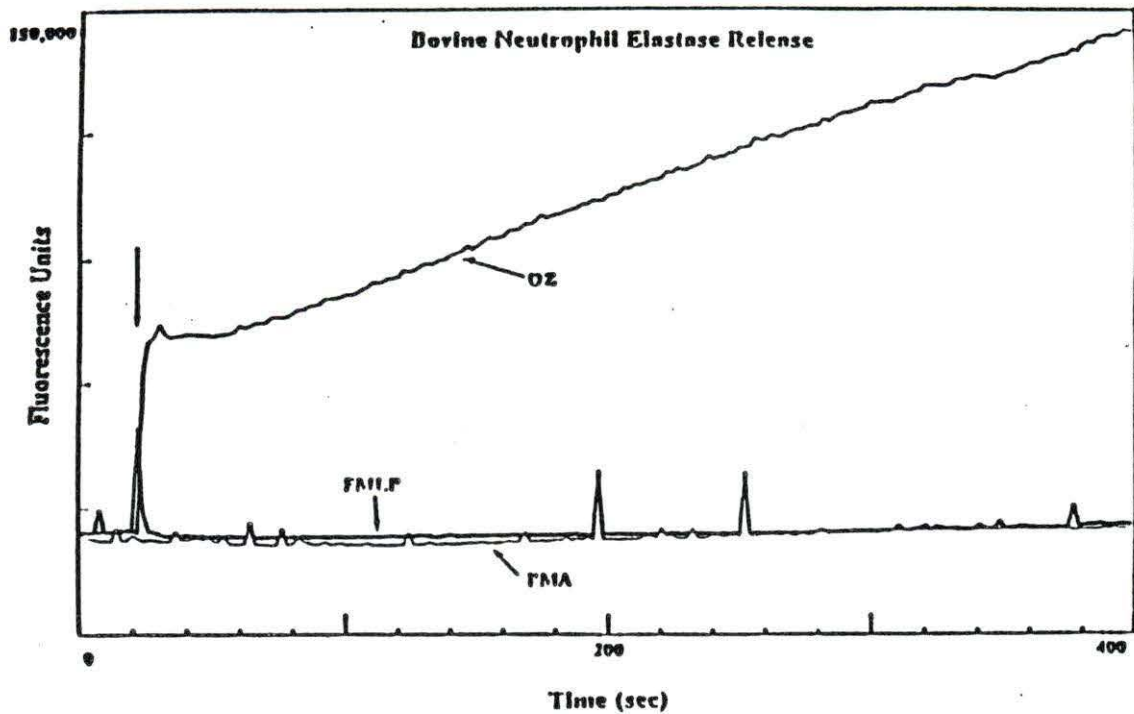


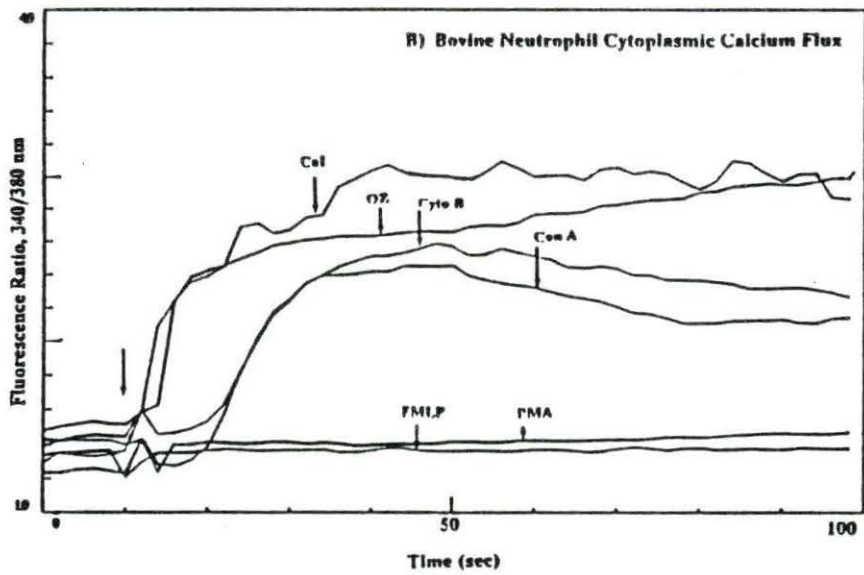
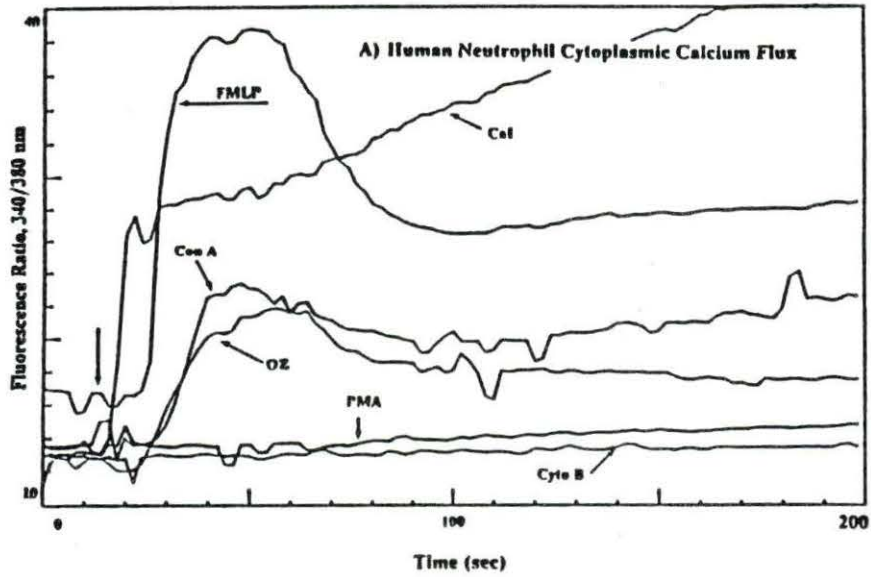
Figure 4. Bovine Neutrophil Elastase Release Stimulated by bOZ, PMA, and FMLP (concentrations were the same as in Fig. 1.). The data are a representative sample and are presented as fluorescence units versus time. A change of 20,000 fluorescence units is equal to 4.1×10^{-11} moles of aminomethylcoumarin formed. The stimulants were added at 20 seconds (indicated by the arrow)

Cytoplasmic calcium flux

An increase in cytoplasmic calcium concentration was detected in human neutrophils when stimulated with calcium ionophore, FMLP, hOZ, and Con A; PMA and cytochalasin B did not stimulate this response (Fig. 5a). Calcium ionophore stimulated a rapid rise in cytoplasmic calcium which continued to gradually increase; this gradual increase may be due to extracellular calcium entering the cell. FMLP, Con A and OZ, which bind to surface receptors, stimulated release of calcium stores into the cytoplasm after a lag period which peaked and decreased. The baseline values for human neutrophils loaded with Fura 2 ranged from 70 - 100 nM calcium and the peak value after stimulation with FMLP was an average of 400 nM calcium. Stimulation of bovine neutrophils with calcium ionophore, OZ, and ConA caused an increase in cytoplasmic calcium concentration; this response was not detected in bovine neutrophils stimulated with PMA (Fig. 5b). In contrast to human neutrophils, cytochalasin B did cause an rise in cytoplasmic calcium in bovine neutrophils, and FMLP did not (Fig. 5). An increase in cytoplasmic calcium in response to cytochalasin B was consistent amongst the animals tested. Even the animals that did not release elastase in response to cytochalasin B did have an increase in cytoplasmic calcium concentration when stimulated with cytochalasin B. Similar to the human neutrophil response, calcium ionophore caused a rapid rise in cytoplasmic calcium; bOZ, cytochalasin B and Con A caused release of cytoplasmic calcium stores after a short lag period. The baseline calcium concentration for bovine neutrophils was similar to the values of human neutrophils, i.e., 70 - 100 nM calcium; the peak value after stimulation with calcium ionophore for bovine neutrophils was an average

stimulation with calcium ionophore for bovine neutrophils was an average of 285 nM calcium.

Figure 5. Increase in Cytoplasmic Calcium. A) Human B) Bovine. The data are a representative sample and are presented as the corrected fluorescence ratio versus time. Each stimulant was added 10 seconds after the initiation of the assay (indicated by the arrow). The stimulants are the same as those defined in Figure 1. The baseline values range from 70 - 100 nM calcium, and the peak value for the bovine neutrophils is 285 nM calcium and for the human neutrophils is 400 nM calcium. These values were determined using the equation described in materials and methods



A summary of the response bovine and human neutrophils to the stimuli tested is presented in Table 1.

Table 1. A summary of responses of bovine and human neutrophils to various stimuli

<u>Stimulant</u>	<u>Elastase Release</u>		<u>Oxidant Production</u>		<u>Cytoplasmic Calcium Increase</u>	
	<u>Bo</u>	<u>Hu</u>	<u>Bo</u>	<u>Hu</u>	<u>Bo</u>	<u>Hu</u>
Phorbol Myristate Acetate	-	+	+	+	-	-
Cytochalasin B	+	-	-	-	+	-
Opsonized Zymosan	+	+ ^a	+	+	+	+
Calcium Ionophore	+ ^a	+ ^a	-	-	+	+
Concanavalin A	+ ^a	+ ^a	-	+	+	+
F-met-leu-phe	-	+ ^a	-	+	-	+

^aPre-incubated with cytochalasin B.

DISCUSSION

Bovine and human neutrophil responses to various stimulants were measured using kinetic assays. Some interesting similarities and differences were observed (Table 1). Cytochalasin B a drug commonly used for pretreatment of human neutrophils was able to stimulate bovine neutrophils to increase cytoplasmic calcium concentration and to release elastase. Phorbol myristate acetate stimulated oxidant production and elastase release by human neutrophils; however, PMA stimulated oxidant production without stimulating elastase release from bovine neutrophils. Bovine neutrophils did not respond to FMLP which was expected since it has previously been reported that the bovine neutrophil lacks FMLP receptors (17). Bovine and human neutrophils also differed in their total elastase activity for the substrate MCA. Human neutrophils when stimulated with Con A had weak oxidant production; this was not observed in bovine neutrophils. Bovine and human neutrophils responded similarly to OZ and calcium ionophore.

Phorbol myristate acetate activates protein kinase C independent of cell surface receptors (31). Stimulation of the oxidative burst and degranulation by PMA in human neutrophils has previously been reported (11,15,42,48). Protein kinase C activity has been shown to be important for the oxidative burst of human neutrophils (11,48), and it has been suggested that PMA stimulation of protein kinase C has more of a role in the oxidative burst than degranulation (40,43). The signal pathways that lead to the production of O_2^- and granule release stimulated by PMA are

probably different since O_2^- production can be inhibited by 1-(5-isoquinoline-sulfonyl)-2-methyl piperazine (H7), a C-kinase inhibitor, but granule release is not (5). Some explanations for how PMA can signal different cellular functions via different pathways have included protein kinase C compartmentalization or different isozymes of protein kinase C regulating the cellular functions (5). The role of protein kinase C in degranulation has not been clearly elucidated. Bovine neutrophils, when stimulated with PMA, produced O_2^- ; however, there was not a detectable release of elastase, even when the cells could be preincubated with cytochalasin B. This may be due to different isozymes of protein kinase C in bovine neutrophils or different proteins phosphorylated resulting in the inability of PMA to stimulate the appropriate signal for elastase release. In bovine neutrophils only the stimulants which caused an increase in cytoplasmic calcium were able to stimulate elastase release; PMA did not stimulate an increase in cytoplasmic calcium. Phorbol myristate acetate's inability to stimulate an increase in cytoplasmic calcium has been previously reported (27). Our data suggest that the activation of protein kinase C by PMA in bovine neutrophils is not important for elastase release. However, elastase was the only granule enzyme measured; therefore, the role of PMA-protein kinase C activation in release of other enzymes cannot be determined.

Cytochalasin B, a microfilament disrupting agent, is commonly used to pretreat neutrophils to promote secretion of granule contents extracellularly upon stimulation (16). However, cytochalasin B pretreatment enhances many human neutrophil responses besides degranulation, and the pretreatment of neutrophils with cytochalasin B has

expanded to many other functional assays (2-4,6,10,12,22,28,32-34,44). The mechanism of cytochalasin B enhancement is not clearly understood (22). In bovine neutrophils loaded with Fura 2, cytochalasin B elicited a detectable increase in cytoplasmic calcium. Also, some samples of bovine neutrophils will release elastase in response to cytochalasin B alone; however, this response was variable. Enzyme release in response to cytochalasin B has been previously reported for guinea pig neutrophils (4). In bovine neutrophils an increase in cytoplasmic calcium appears to be required for the release of elastase; however, the elevation of calcium alone is not sufficient to elicit elastase release. For example, cytochalasin B consistently caused an increase in cytoplasmic calcium, but the release of elastase in response to cytochalasin B was variable. Human neutrophils differ in that elastase is released without an increase in cytoplasmic calcium when the cell is stimulated with PMA.

Zymosan opsonized with fresh human or bovine serum stimulated a strong and consistent response in all three assays in both species tested. Opsonized zymosan is a particulate stimulant that binds to surface receptors and stimulates a receptor mediated signal transduction pathway (36). This stimulant elicited the most consistent responses from bovine neutrophils of the stimulants tested. The particulate stimulants of neutrophils can elicit a "frustrated phagocytic" response which means the neutrophil secretes some of its lysosomal enzymes extracellularly in an effort to phagocytize the particle. The particulate stimulant is, therefore, a good stimulant for use with bovine neutrophils since soluble stimuli require the neutrophils to be pretreated with cytochalasin B in order to consistently get degranulation to the outside of the cell, and

cytochalasin B itself can cause the release of lysosomal enzymes. Opsonized zymosan alone stimulated human neutrophils to release elastase; this response was delayed compared to the response of cells pretreated with cytochalasin B.

Calcium ionophore, A23187, facilitates calcium entry into the neutrophil independent of surface receptors (7,29). Concanavalin A is a lectin that binds to surface receptors (26). Concanavalin A stimulated weak oxidant production in human neutrophils, but not in bovine. The reason for this difference is unknown. A strong O_2^- response to Con A in human neutrophils preincubated with cytochalasin B has been reported (25). Calcium ionophore and Con A stimulate an increase in cytoplasmic calcium and release of elastase in human neutrophils pretreated with cytochalasin B. This response was similar in bovine neutrophils pretreated with cytochalasin B. The problem with using these soluble stimulants to measure elastase release in bovine neutrophils is the inability to consistently pretreat with cytochalasin B. Bovine neutrophils may be stimulated to degranulate in response to the increased cytoplasmic calcium, but they may not be releasing their granule contents extracellularly without cytochalasin B pretreatment.

Formyl-methionyl-leucyl-phenylalanine, a synthetic analogue of bacterial peptides, binds to surface receptors on human neutrophils (13). FMLP stimulated oxidant production, elastase release, and cytoplasmic calcium increases in human neutrophils. There was no response to this stimulant by bovine neutrophils; it has previously been reported that bovine neutrophils do not have FMLP receptors (17).

The total elastase activity for the substrate MCA was determined for bovine and human neutrophils. The human neutrophils had about 50 times more total elastase activity compared to the bovine neutrophils. This difference may be due to total elastase content of the bovine neutrophils, or it may be due to a difference in the activity of bovine elastase for the substrate used. There are several other enzymes associated with human neutrophil azurophilic granules that have low activity in bovine neutrophils when compared to human neutrophils (14). In addition, the elastase activity was determined 5 minutes after the cells were lysed; the role of elastase inhibitors contributing to decreased enzyme activity was not determined. It has been reported that bovine neutrophils contain highly active cytosolic elastase inhibitors (21,30).

In summary, bovine neutrophils respond differently than human neutrophils to PMA, cytochalasin B, Con A and FMLP. They differ in their response to stimulation of protein kinase C by PMA, and, in bovine but not human neutrophils, there is a correlation of increased intracellular calcium and elastase release. In addition, the use of zymosan opsonized with homologous serum as a stimulant allows the evaluation of either human or bovine neutrophil oxidant production, elastase release, and intracellular calcium fluxes without the need for cytochalasin B pretreatment.

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SECTION III.

DEFECTIVE FUNCTION IN NEUTROPHILS AND LYMPHOCYTES
FROM CATTLE PERSISTENTLY INFECTED WITH
BOVINE VIRAL DIARRHEA VIRUS
AND THE INFLUENCE OF RECOMBINANT BOVINE INTERFERON
GAMMA AND INTERLEUKIN 2

ABSTRACT

Cattle persistently infected with bovine viral diarrhea (BVD) virus have decreased neutrophil and lymphocyte function. This investigation reevaluated these functions and further characterized the inhibition of persistent BVD virus infection in neutrophils using sensitive kinetic assays. In addition, the influence of *in vitro* incubation of neutrophils with recombinant bovine interferon gamma (rboIFN gamma) and *in vitro* incubation of lymphocytes with recombinant bovine interleukin 2 (rboIL2) was evaluated.

A significant ($P < 0.05$) decrease in random migration under agarose, Staphylococcus aureus ingestion, cytochrome C reduction, iodination, antibody-independent cell-mediated cytotoxicity (AINC), oxidant production, and cytoplasmic calcium flux were observed in neutrophils from cattle persistently infected with BVD virus when compared to noninfected controls. Incubation of neutrophils from noninfected controls with rboIFN gamma significantly decreased random migration under agarose, cytochrome C reduction, and cytoplasmic calcium flux. Neutrophils from persistent BVD virus infected cattle also exhibited decreased random migration under agarose after incubation with rboIFN gamma; in addition, AINC, elastase release and cytoplasmic calcium flux were significantly enhanced. Recombinant bovine IFN gamma induced significantly ($P < 0.05$) different effects on chemotaxis, cytochrome C reduction, iodination and cytoplasmic calcium flux of neutrophils from infected and control animals. The rboIFN

gamma was much more effective at improving the function of neutrophils from cattle persistently infected with BVD than in controls.

Lymphocytes from infected cattle exhibited decreased blastogenesis in response to phytohemagglutinin (PHA), concanavalin A (ConA), and pokeweed mitogen (PWM). Incubation of those lymphocytes with rboIL2, with no mitogen present, significantly increased the incorporation of tritiated thymidine. However, the response of lymphocytes to mitogen stimulation was not significantly increased by the presence of rboIL2, indicating that the depression of lymphocyte blastogenesis is not due to a decreased production of IL2 in vitro.

INTRODUCTION

Bovine viral diarrhea (BVD) virus is classified as a pestivirus in the Togaviridae family. The virus is ubiquitous in the cattle population of North America and virulent in its cytopathic and noncytopathic forms. Bovine viral diarrhea has many clinical manifestations including: mild or subclinical infection (the most common form), persistent infection, mucosal disease, and chronic BVD (2,15,29).

Persistent BVD (BVD) infection occurs when a noncytopathic virus infects the fetus before 125 days of gestation (before the fetus is immunocompetent). The fetus becomes immunotolerant to the virus and does not produce antibodies to it (21). At birth, persistently infected calves have a constant viremia and serve as natural reservoirs of the virus (3,21). Clinical signs of persistent BVD virus infection include decreased weight gain and stunted growth; however, many persistently infected calves grow normally and do not show clinical signs of infection (3,12,21).

Mucosal disease is induced when an animal persistently infected with noncytopathic BVD virus is co-infected with an appropriate cytopathic BVD virus (8,11). Mucosal disease is characterized by gastrointestinal abnormalities, i.e., oral lesions and profuse diarrhea. The virus has an affinity for the cells of the lymphoid tissue and often a leukopenia and neutropenia are observed. It is not known whether the leukopenia and neutropenia are due to viral infection of the bone marrow, to soluble factors which have an effect on the bone marrow, or to destruction of

lymphocytes and neutrophils. Death from mucosal disease usually occurs within 3 to 10 days of onset of clinical signs (2,15,29). Chronic BVD is also a disease of high mortality. However, in chronic BVD there is severe emaciation, lameness and intermittent or constant diarrhea. The animal may survive up to 18 months (2,9,15,29).

The BVD virus is immunosuppressive in cattle, affecting the function of several cell types. Similar to other viral infections, animals infected with BVD virus have decreased circulating lymphocyte and neutrophil numbers (7,16,36). However, the immunosuppression is not entirely a result of decreased numbers. The immunosuppression observed with BVD infection includes decreased mitogen-induced lymphocyte blastogenesis (22,23,27,32,35), decreased monocyte chemotaxis (19), decreased ingestion of S. aureus by neutrophils (32), decreased iodination (myeloperoxidase-H₂O₂-halide activity) in neutrophils (35,36), and decreased antibody-dependent cell-mediated cytotoxicity (ADCC) by neutrophils (35). The basic mechanism of this viral induced immunosuppression is not clear, but it predisposes the animal to secondary bacterial infections or other viral infections (13,28,30).

The specific interest in this investigation was the immunosuppression observed in persistent BVD virus infection. It has been shown that persistently infected cattle have decreased lymphocyte blastogenesis and decreased S. aureus ingestion by neutrophils (32). The primary objective of this investigation was to better characterize the suppression of neutrophil function in cattle with persistent BVD infection. Using kinetic assays for studying neutrophil function, we were able to better define the suppressive effects of persistent BVD infection on neutrophils.

Our second objective was to determine the effect of in vitro recombinant bovine interferon gamma (rboIFN gamma) on the function of neutrophils and the effect of recombinant bovine interleukin 2 (rboIL2) on blastogenesis of peripheral blood lymphocytes from cattle persistently infected with the virus.

MATERIALS AND METHODS

Animals

Nine healthy 1 - 2 year old holstein steers housed at Iowa State University served as controls. Nine cattle persistently infected with BVD virus (ages 1 - 6 years, 6 females and 3 steers) were part of the herd at the National Animal Disease Center, Ames, IA. Four of the cattle were persistently infected with virus isolate TGAN, and four of the cattle were persistently infected with virus isolate NEB. These cattle were experimentally infected during gestation. The other animal was infected with virus isolate 9789, and was detected as being naturally persistently infected at 6 months of age; there was no clinical evidence of the infection.

Lymphokine Preparation

Recombinant bovine interferon gamma was supplied by CIBA-GEIGY, Limited, Basel, Switzerland. The preparation (lot number 322/38) had 12.5 mg protein/ml and a specific activity of approximately 2.2×10^6 Units/ml. Recombinant bovine interleukin 2 was supplied by Immunex Corp., Seattle, Washington. The preparation (lot number 21309910) had 1.99 ug protein/ml and a specific activity of approximately 20,000 Units/ml.

Neutrophil Isolation

Bovine neutrophils were isolated as previously described (34). Briefly, 250 ml of peripheral blood from control animals and 500 ml of peripheral blood from the persistently infected animals were collected into acid citrate dextrose anticoagulant. After centrifugation the plasma was discarded and the buffy coat was removed and used to isolate lymphocytes. The packed red blood cells were lysed with cold buffered hypotonic solution for one minute and then isotonicity was restored. The cells were pelleted and the lysing step was repeated. The isolated neutrophils (purity greater than 90 percent) were resuspended in phosphate buffered saline (PBSS) (0.015 M) at a concentration of 1.0×10^8 cells/ml. Five hundred microliters (ul) of the cell suspension were incubated for 2 hours with an equal volume of medium 199 (M199, Gibco, Grand Island, NY) with or without rboIFN gamma (CIBA-GEIGY Limited, Basel, Switzerland). The final concentration of rboIFN gamma on the neutrophils was 5.0×10^{-9} g/ml.

Total White Blood Cell Counts

Total white blood cell counts were determined for the persistently infected and the control cattle using an electronic cell counter. These values were determined on 5 different days.

Kinetic Assays of Neutrophil Function

These assays were performed on an SIM 8000C photon-counting spectrofluorometer (SIM Instruments, Inc., Urbana, IL). The neutrophil stimulant used in all assays was zymosan A (Sigma Chemical Co., St. Louis, MO) opsonized with fresh bovine serum (OZ), as previously described (34). Opsonized zymosan was used at a final concentration of 1.0 mg/ml.

Oxidant production assay

This assay, a modification of the procedure described by Hyslop and Sklar (1984), indirectly measures O_2^- production by neutrophils, as previously described (10). When neutrophils are appropriately stimulated and O_2^- is produced, it is rapidly converted to H_2O_2 in the presence of superoxide dismutase (SOD). The H_2O_2 , in the presence of horseradish peroxidase (HP), oxidizes p-hydroxyphenylacetate (PHPA) to a fluorescent product $PHPA_2$. $PHPA_2$ emits light at a 400 nm wavelength when excited by light at a 340 nm wavelength. Each cuvette contained 2.5 ml HBSS without phenol red (Gibco), 5.0×10^6 neutrophils, and 75 μ l of a reagent cocktail consisting of SOD (Sigma) (8.0 mg/ml PBS), HP (Sigma) (8.0 mg/ml PBS), and PHPA (Sigma) (10.0 mg/ml PBS) at a ratio of 10:10:25, respectively. This assay was run using a program which allowed us to test 10 samples simultaneously taking fluorescence readings of each sample every 150 seconds for 600 seconds. This included a baseline reading and then a 0 time reading which was the fluorescence just after the stimulant was added.

Elastase release assay

Elastase is an enzyme contained in neutrophil granules. The procedure described by Sklar et al. (1982) for measuring elastase release was modified as previously described (10). This enzyme acts on the substrate methylsuccinylalanylalanylprolylvalylaminomethyl-coumarin (MCA), which when cleaved by elastase liberates a fluorescent product aminomethylcoumarin (AMC). Aminomethylcoumarin when excited by light at 380 nm wavelength will fluoresce at 490 nm wavelength. Each cuvette for this assay contained 2.5 ml HBSS, 5.0×10^6 neutrophils, and 75 μ l of MCA. The MCA (Peninsula Laboratories) was dissolved in dimethylsulfoxide (DMSO) and then diluted in PBS to a stock concentration of 1.25 mg/ml and stored at -20°C . This assay was performed using a program as described for the oxidant assay.

Cytoplasmic calcium fluxes

Fura 2-acetoxymethylester (AM) is a fluorescent calcium indicator used to determine cytoplasmic calcium fluxes, as previously described (10). The neutrophils were incubated with Fura 2-AM for 30 minutes. During the incubation the Fura 2-AM enters the cell and is hydrolysed by intracellular enzymes thus trapping the Fura 2 inside the cells. The peak absorbance of Fura 2 shifts from 380 nm to 340 nm wavelength upon binding calcium. Therefore, the ratio of free versus bound calcium can be measured during activation of the neutrophil by OZ. The SLM 8000C alternates the excitation wavelength between 340 nm and 380 nm every two seconds and records the emittance at 510 nm wavelength. The corrected

ratio of fluorescence (340nm/380nm) is stored by the computer. Each cuvette contained 2.5 ml HBSS and 5.0×10^6 Fura 2 loaded neutrophils. The cuvette was placed into the sample chamber and the cells were stimulated 10 seconds after the initiation of the assay. Fluorescence ratios were recorded every two seconds for 100 seconds. Calcium concentration can be determined from the fluorescence ratio as previously described (17).

Other neutrophil function assays

Additional assays were performed to evaluate neutrophil function in cattle persistently infected with BVD virus and the effect of in vitro incubation with rboIFN gamma. These assays were performed as previously described (20,32). Briefly, random migration under agarose was measured after an incubation period of 18 hours; the area of random migration was reported in square millimeters (mm^2). Chemotaxis was measured by migration under agarose toward zymosan activated serum; the chemotactic index was determined by dividing the distance of directed migration by the distance of random migration. Phagocytosis was measured using antibody-coated ^{125}I -iododeoxyuridine-labeled S. aureus. Neutrophils were incubated for ten minutes with bacteria at a ratio of 60:1 (bacteria to neutrophil) and then lysostaphin was added to remove the extracellular S. aureus; the results were reported as percent ingested. The reduction of cytochrome C, a measure of superoxide anion production, was evaluated after a 30 minute incubation period of neutrophils with cytochrome C and OZ. The results were reported as the optical density/ 1.25×10^6 PMN/30 minutes. The iodination reaction, a measure the myeloperoxidase- H_2O_2 -

halide system, was performed by incubating neutrophils with OZ and Na^{125}I for 20 minutes; the reaction was terminated by the addition of trichloroacetic acid. The results were reported as nmole $\text{NaI}/10^7$ PMNs/hour. Antibody-dependent cell-mediated cytotoxicity was evaluated using antibody coated ^{51}Cr -labeled chicken red blood cells as the target cell. The effector to target cell ratio was 10:1, and the results are reported as the percent of specific release which occurred during a two hour incubation. Antibody-independent cell-mediated cytotoxicity was measured similar to ADCC; however, antibody was not added.

Lymphocyte Evaluation

Lymphocytes were isolated from peripheral blood, and lymphocyte blastogenesis was performed using pokeweed mitogen (PWM), phytohemagglutinin (PHA), and concanavalin A (Con A) as mitogens, with a 72-hour incubation period, as previously described (35). In addition, rboIL2 was evaluated in vitro for its effect on the blastogenesis of lymphocytes from cattle persistently infected with BVD virus and controls. The lymphocytes were incubated with or without 10.0 ng/ml rboIL2 (Immunex Corp., Seattle, Washington)

Statistical Analysis

Data were analyzed using the Statistical Analysis System (SAS Institute, Inc., Cary, NC). The analysis of variance was performed using a split plot experimental design. With BVD infection status as the whole plot and lymphokine (rboIFN gamma or rboIL2) treatment as the subplot.

Date of assay was used as a blocking factor for all assays and, in addition, time point of data collection was used as a blocking factor for the kinetic neutrophil assays. The main effects of BVD virus infection and in vitro treatment with lymphokine (rboIFN gamma or rboIL2) were determined. Also, the interaction between virus infection status and lymphokine treatment was evaluated. Since a significant interaction was noted in a number of instances the direct effect of lymphokine treatment on cells from either control or BVD infected animals was also evaluated using a one-way analysis of variance with a split plot design.

RESULTS

Total White Blood Cell Counts

The WBC counts in the persistently infected cattle were consistently lower than the controls. In the control group the values ranged from 4,400 cells/ul to 14,000 cells/ul with a mean of 8,700 cells/ul and average differential WBC counts of 41% neutrophils, 58% lymphocytes, and 2% eosinophils compared to the cattle persistently infected with BVD virus which ranged from 2,520 cells/ul to 9,900 cells/ul with a mean of 4,300 cells/ul and average differential WBC counts of 22% neutrophils, 76% lymphocytes, and 2% eosinophils.

Neutrophil Assays

The results from assays examining the effects of persistent BVD virus infection, rboIFN gamma and the interaction of these two on neutrophil functions are shown in Tables 1-2, and Figures 1-3. When compared to controls, neutrophils from cattle persistently infected with BVD virus had significantly ($P < 0.05$) decreased random migration under agarose, ingestion of S. aureus, cytochrome C reduction, iodination, AINC, oxidant production, and intracellular calcium flux. In addition, there was a tendency ($P = 0.06$) for elastase release to be decreased.

Table 1. Bovine neutrophil assay values (means) for four treatment groups and the level of significance for the main effects of PBVD^a virus infection and rboIFN gamma and their interaction using an analysis of variance procedure

PMN function	Treatment Group Assay Values				SEM
	Control (n=18)	Control plus rboIFN gamma (n=18)	PBVD ^a (n=18)	PBVD ^a plus rboIFN gamma (n=18)	
Random migration (mm ²)	60	44	48	38	±3
Chemotaxis (ratio)	1.33	1.36	1.44	1.25	±0.04
<i>S. aureus</i> ingestion (%)	29	31	21	24	±1
Cytochrome C reduction (OD)	0.87	0.80	0.74	0.80	±0.02
Iodination (nmol NaI/10 ⁷ PMN/hr)	34	31	25	27	±1
Antibody-independent cell- mediated cytotoxicity (%)	16	18	2	8	±1
Antibody-dependent cell- mediated cytotoxicity (%)	52	57	48	54	±3

^aPBVD = cattle persistently infected with BVD virus.

Table 2. The level of significance for the effects of PBVD^a virus infection and rboIFN gamma on neutrophils and their interaction using an analysis of variance procedure

PMN function	Control vs PBVD	Control vs Control plus rboIFN gamma	PBVD ^a vs PBVD ^a plus rboIFN gamma	PBVD ^a by rboIFN gamma interaction
Random migration	0.048	0.001	0.03	0.57
Chemotaxis ratio	0.71	0.13	0.08	0.02
<i>S. aureus</i> ingestion	0.001	0.44	0.25	0.99
Cytochrome C reduction	0.001	0.01	0.13	0.001
Iodination	0.03	0.50	0.37	0.01
Antibody-independent cell-mediated cytotoxicity	0.002	0.69	0.02	0.22
Antibody-dependent cell-mediated cytotoxicity	0.22	0.33	0.17	0.65
Oxidant Production	0.03	0.79	0.16	0.19
Elastase Release	0.06	0.14	0.002	0.72
Cytoplasmic Ca ⁺⁺ Increase	0.01	0.02	0.001	0.003

^aPBVD = cattle persistently infected with BVD virus.

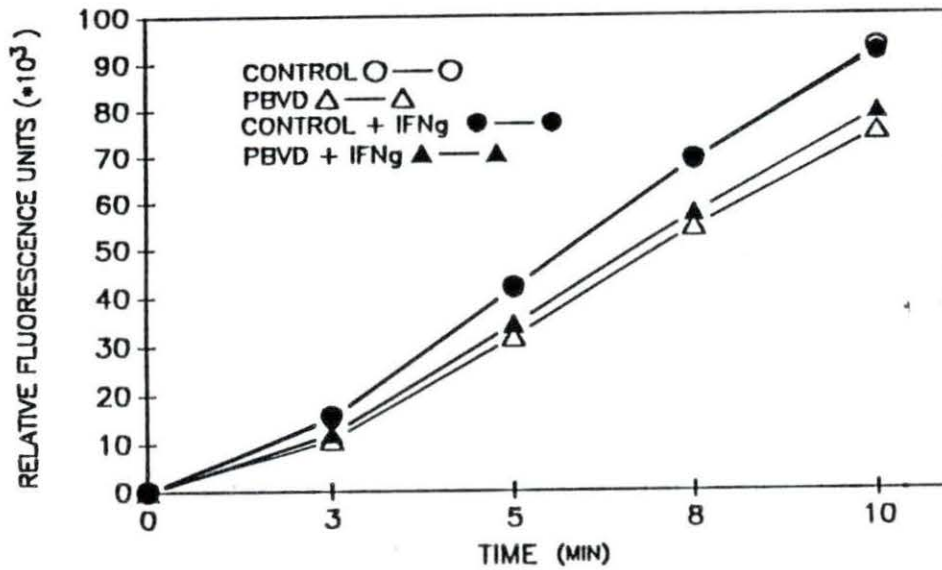


Figure 1. Mean values ($n=18$) for the kinetic assay measuring oxidant production by neutrophils from four treatment groups. The mean values are the change in fluorescence units from the baseline at time 0. The SEM for these means is 0.6×10^3 . PBVD = cattle persistently infected with BVD; IFNg = recombinant bovine IFN gamma

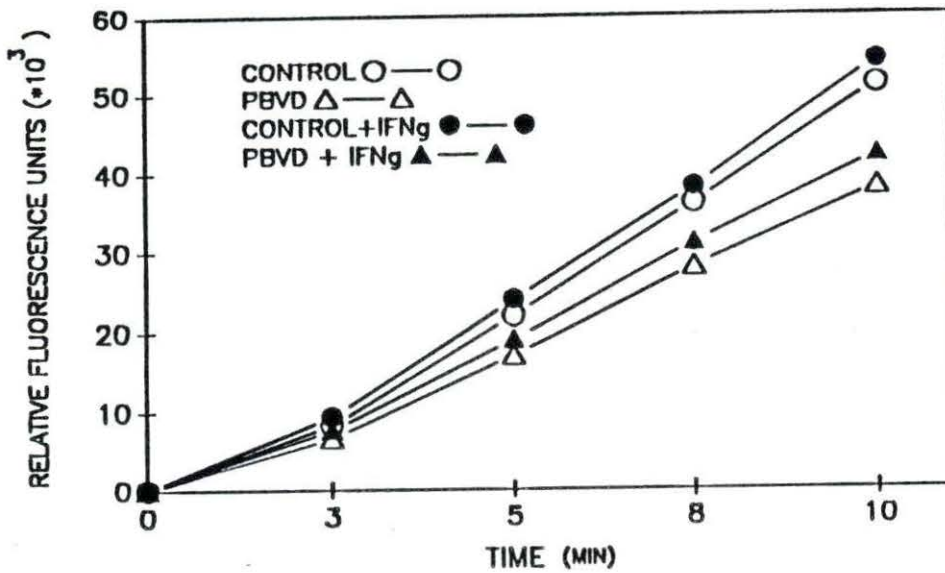


Figure 2. Mean values ($n=18$) for the kinetic assay measuring elastase release from bovine neutrophils in four treatment groups. The mean values are the change in fluorescence units from the baseline at time 0. The SEM for these means is 0.4×10^3 . PBVD = cattle persistently infected with BVD virus; IFNg = recombinant bovine IFN gamma

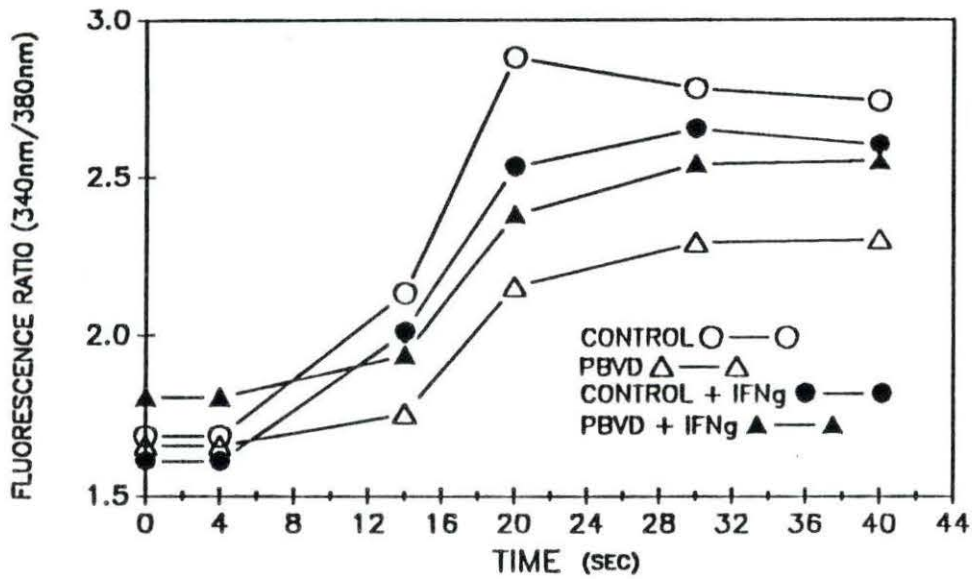


Figure 3. Mean values for the kinetic assay measuring cytoplasmic calcium fluxes in neutrophils from four treatment groups. These mean values represent the corrected fluorescence ratio (Ex 340 nm/380 nm). The SEM for these means is 0.13. PBVD = cattle persistently infected with BVD virus; IFNg = recombinant bovine IFN gamma

Incubation of neutrophils from control animals with rboIFN gamma resulted in significantly ($P < 0.05$) decreased random migration under agarose, cytochrome C reduction, and cytoplasmic calcium flux (Tables 1-2, Fig. 3).

Incubation of neutrophils from persistently infected cattle with rboIFN gamma resulted in a significant ($P < 0.05$) decrease in random migration under agarose, and increase of the following functions: AINC, elastase release, and cytoplasmic calcium flux (Tables 1-2, Figs. 2-3).

The interaction between persistent virus infection and rboIFN gamma was statistically evaluated; the results are shown in Table 2. A significant ($P < 0.05$) interaction was observed between persistent infection and in vitro incubation with rboIFN gamma for chemotaxis, cytochrome C reduction, iodination and cytoplasmic calcium flux. After incubation in rboIFN gamma, neutrophils from cattle persistently infected with BVD had decreased chemotaxis and increased functions of cytochrome C reduction, iodination and cytoplasmic calcium flux. The influence of rboIFN gamma on neutrophils from the control group was the opposite.

Lymphocyte Blastogenesis

Results of the lymphocyte blastogenesis are shown in Table 3. Lymphocytes from persistently infected cattle exhibited significantly ($P < 0.05$) decreased response to mitogen stimulation by PHA, ConA, and PWM. Incubation media which included rboIL2 significantly ($P < 0.01$) increased the background counts of lymphocytes of control and persistently infected cattle but did not increase the mitogen stimulated responses (Table 3).

Significant interaction between *rboIL2* and persistent BVD virus infection was not detected.

Table 3. Tritiated thymidine uptake by resting or stimulated peripheral blood lymphocytes from four treatment groups and the level of statistical significance of the effects of PBVD^a virus infection and rboIL2 on lymphocytes and their interaction using an analysis of variance procedure

Mitogen	Treatment Group Assay Values					Probability of > F			
	Control (n=17) C _{pm} ^b	Control plus rboIL2 (n=16) cpm ^b	PBVD ^a (n=17) cpm ^b	PBVD ^a plus rboIL2 (n=16) cpm ^b	SEM	Control vs PBVD ^a	Control vs Control plus rboIL2	PBVD ^a vs PBVD ^a plus rboIL2	PBVD ^a by rboIL2 interaction
None	700	4600	500	6600	± 600	.08	.0001	.0001	.10
PHA	23700	27400	14100	15300	±2500	.01	.38	.81	.90
Con A	49100	54000	21600	22400	±2400	.0001	.35	.91	.91
PWM	17300	16600	6000	6600	± 700	.0001	.64	.67	.29

^aPBVD = cattle persistently infected with BVD.

^bcpm = counts per minute.

DISCUSSION

This investigation extends the results of a previous study defining alterations of neutrophil and lymphocyte function in cattle persistently infected with BVD virus (32). Additional aspects of neutrophil function were found to be suppressed, and in vitro incubation with rboIFN gamma was shown to significantly ($P < 0.05$) improve several of the suppressed functions. In vitro incubation with rboIL2 was not able to improve the depression of lymphocyte blastogenesis in animals persistently infected with BVD virus; indicating the depression was not due to a decreased production of IL2 in vitro.

The influence of interferon gamma on neutrophils has been evaluated. Human neutrophils incubated with recombinant human IFN gamma (rhuIFN gamma) have an increased number of Fc receptors and enhanced phagocytic and cytotoxic functions (4,25,37,38). Interferon gamma (in vitro and in vivo) alters neutrophil functions of cattle; although the results are variable (5,33,40,41). The reason for this variability is not clear. In general, rboIFN gamma improves the function of neutrophils with suppressed function more than neutrophils from healthy control cattle (33,40), as was found in this study. Therefore, the variability observed in the effects of rboIFN gamma on neutrophil function from normal cattle may be due to variability in physiologic status of clinically healthy animals.

The mechanism of action of IFN gamma is not well described; however, it is known that IFN gamma binds to a cell surface receptor (43,44). Also, protein synthesis and arachidonic acid metabolism by neutrophils are

needed for IFN gamma to modulate some, but not all, of the bovine neutrophil functions (41).

Incubation of neutrophils from animals persistently infected with BVD virus with recombinant boIFN gamma significantly enhanced AINC, elastase release and cytoplasmic calcium flux, and inhibited random migration in this study. Incubation of neutrophils from control animals with rboIFN gamma resulted in significantly ($P < 0.05$) inhibited random migration under agarose; however, cytochrome C reduction and cytoplasmic calcium flux were decreased. Significant ($P < 0.05$) interactions between rboIFN gamma and persistent BVD virus infection were detected for neutrophil chemotaxis, cytochrome C reduction, iodination and cytoplasmic calcium increase. This indicates that the effect of rboIFN gamma on neutrophils from healthy cattle is significantly different from the effect on neutrophils from cattle with persistent BVD virus infected cattle for these assays.

The mechanisms for the decreased cytoplasmic calcium flux and decreases noted in other neutrophil functions of cattle persistently infected with BVD is unknown. Possibly, a decrease in cell surface receptors or an alteration in one of many steps in signal transduction is involved.

The signal transduction pathway that occurs after an opsonized particle binds to a bovine neutrophil has not been clearly defined. However, some possible explanations for the defects observed in neutrophils of persistently infected cattle can be made based on what is known of the signal transduction pathway of human neutrophils (24). Briefly, an opsonized particle binds to a receptor that signals a G

protein. The G protein activates an enzyme that cleaves membrane lipids resulting in the formation of a cytosolic messenger (inositol triphosphate, IP3) and a lipid soluble messenger (diacylglycerol, DAG). The cytosolic portion signals calcium to be released from intracellular stores and the membrane portion stimulates protein kinase C. The importance of the cytoplasmic calcium flux has been reported for many neutrophil functions. The calcium flux is one of the initial events in receptor-mediated activation, and is essential for subsequent cellular responses (10,24,39). It is likely that the decreased cytoplasmic calcium flux in neutrophils from cattle persistently infected with BVD virus contributes to the other defective functions. This is somewhat supported by the concurrent improvement in cytoplasmic calcium flux and several other functions of neutrophils from persistently infected cattle following incubation with rboIFN gamma. Recombinant boIFN gamma may reverse the defective step(s) in the signal transduction pathway or act at another step.

The lymphocytes from cattle persistently infected with BVD virus had decreased lymphocyte blastogenesis in response to all three mitogens when compared to controls. These results are consistent with previous reports of decreased blastogenesis in response to mitogens by persistent BVD virus infected cattle (32). Other forms of BVD virus infection are reported to result in decreased lymphocyte blastogenic response to mitogens, also (22,23,26,27,31,35). The mechanism for the decrease in blastogenesis is not known.

Interleukin 2 is important for proliferation of T cells. Interleukin 2 binds to a receptor that activates protein kinase C resulting in

phosphorylation of proteins leading to DNA synthesis (1). One possible cause for the decrease in lymphocyte blastogenesis of cattle persistently infected with BVD virus is decreased production and/or secretion of IL2 from T helper cells. It has been shown that the addition of IL2 to lymphocyte cultures reverses the suppression of lymphocyte blastogenesis induced by cortisol (6).

In this investigation, rboIL2 was mitogenic by itself as seen by its influence on lymphocyte proliferation in the absence of mitogens for lymphocytes from both healthy cattle and cattle persistently infected with BVD. The fact that blastogenesis was equally enhanced in both groups by rboIL2 in the absence of mitogens suggests that the lymphocyte defect is not in the IL2 stimulatory pathway. The enhancement of lymphocyte blastogenesis by IL2 in unstimulated lymphocytes has been previously described (1,14). An additive effect on blastogenesis was seen when the lymphocytes from control cattle were incubated with both rboIL2 and either PHA or Con A (Table 3); although, this effect was not statistically significant. This is consistent with a previous report that optimal concentrations of Con A and recombinant human IL2 had an additive effect on bovine lymphocyte blastogenesis (42). However, this additive effect was not observed in the lymphocyte cultures from persistently infected cattle even though the rboIL2 significantly increased the background counts. The cause for this lack of additive effect of rboIL2 and PHA or Con A on lymphocytes from persistently infected animals is not known, but it is probably due to the same factors that limit lymphocyte proliferation in response to mitogens. The addition of rboIL2 to lymphocyte cultures did not significantly enhance the blastogenic response of these cells to

mitogens. This suggests that a deficiency of IL2 is not responsible for the decreased blastogenic responsiveness of lymphocytes from infected animals. The mechanism of suppression of lymphocyte blastogenesis is not known. However, since neutrophils and lymphocytes use a similar signal transduction pathway (18), it may be that a similar defect in signal transduction is responsible for the inhibition of both lymphocyte and neutrophil function.

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GENERAL SUMMARY AND DISCUSSION

The purpose of this research was to further characterize the suppression of neutrophil function from cattle persistently infected with BVD virus, and to examine the effect of rboIFN gamma and rboIL2 on the function of neutrophils and lymphocytes from these cattle. The results of this work add to the body of information on suppression of bovine neutrophils and lymphocytes and the enhancement of function by recombinant cytokines in infectious diseases of cattle.

The first objective of this research was to adapt fluorometric kinetic neutrophil functional assays for use in bovine neutrophil research. The fluorometric assays measure the oxidative burst, elastase release and cytoplasmic calcium flux. These assays have been used for examination of human but not bovine neutrophils (5,6,11). A comparison was made of the response of human and bovine neutrophils to various neutrophil stimuli using these assays. Several interesting observations were made. Cytochalasin B is a drug commonly used to pretreat neutrophils to inhibit microfilaments forcing stimulated neutrophils to release granule enzymes extracellularly (4). In bovine neutrophils, the cytochalasin B alone caused elastase to be released extracellularly from granules. The results of elastase release in bovine neutrophils in response to cytochalasin B is variable; the reason for the variability is unclear. In addition, the cytochalasin B stimulated an increase in intracellular calcium. This response was not observed in human neutrophils. Another interesting comparison is the response of human and

bovine neutrophils to phorbol myristate acetate. PMA stimulates protein kinase C independent of cell surface receptors (8). Human neutrophils were stimulated by PMA to release elastase and to produce superoxide anion without a detectable increase in cytoplasmic calcium (7,12,13). Bovine neutrophils produced superoxide anion in response to PMA and did not have a detectable increase in cytoplasmic calcium; however, there was no detectable release of elastase in response to PMA. Another difference observed was the total elastase activity of human and bovine neutrophils. Bovine neutrophils have been reported to have much less activity than human neutrophils for several other granule enzymes (3). Concanavalin A weakly stimulated superoxide anion production in human neutrophils but not bovine. The response to zymosan opsonized with fresh serum was similar in bovine and human neutrophils for all three assays. Opsonized zymosan (OZ) is a particulate stimuli and binds to the C3b receptor; the responses to OZ were consistent.

The reason for the differences in the response to various stimuli is unclear. The signal transduction pathway for bovine neutrophils has not been thoroughly investigated. It will be interesting to compare the bovine and human neutrophils once those details are known.

Using these assays in addition to the traditional neutrophil functional assays, the defective neutrophil function in cattle persistently infected with BVD was further characterized. In addition, the effect of recombinant cytokines (rboIFN gamma and rboL2) on neutrophil and lymphocyte functions was examined.

In this research, additional neutrophil functions were observed to be suppressed in cattle persistently infected with BVD virus. Consistent

with previous reports, lymphocyte blastogenesis in response to Con A, PHA and PWM was suppressed.

Incubation of neutrophils with rboIFN gamma was able to enhance many of the suppressed functions. Cytoplasmic calcium flux is an important initial event in neutrophil function; this response is suppressed in neutrophils from cattle persistently infected with BVD. Incubation with rboIFN gamma improved the cytoplasmic calcium flux of neutrophils from cattle persistently infected with BVD virus. It is possible that improved cytoplasmic calcium flux contributed to the improvement of other functions. The reason for enhanced cytoplasmic calcium flux was not determined; it may be related to receptor numbers or any other step along the signal transduction pathway resulting in increased cytoplasmic calcium concentration.

Lymphocyte blastogenesis was not improved by the incubation of lymphocytes with rboIL2. The rboIL2 had some mitogenic activity itself, but the response to rboIL2 and mitogen was not additive. This suggested that the suppression of function was not due to an inability to produce or secrete IL2, and the defect is still unknown.

Based on human literature, neutrophils and lymphocytes use similar signal transduction pathways, and it is possible that the defect in cattle persistently infected with BVD is similar in the two cell types (1,2,9). Certain obvious areas to examine include receptor, number and affinity, formation of products along the signal transduction pathway, and the calcium flux in lymphocytes.

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