Characterization of neutrophil function in young calves

by

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GENERAL INTRODUCTION

Young calves have an increased susceptibility to enteric and respiratory disease (Bryson et al. 1978; Roy et al. 1980). Decreased function of neutrophils observed in young calves (Hauser et al. 1986) might help to explain the increased morbidity. Understanding the biochemical and molecular basis for neutrophil dysfunction in young animals is important for designing methods to improve neutrophil function.

Cytokines have been shown to have potent modulating activity on neutrophils both *in vitro* and *in vivo*. The use of cytokines to improve neutrophil function has been proposed as a method to decrease the susceptibility of young animals to infectious diseases.

Explanation of Thesis Format

A review of the literature on neutrophil function in immature animals is presented following the general introduction. Paper I presents the results of a study comparing neutrophil function between two age groups (young and adult) of cattle and their response to IFN-γ. Work on the effect of genistein on neutrophil function to examine the role of protein tyrosine kinase(s) is presented in Paper II followed by a general summary.

The work in Paper I has been published in *Comparative Haematology International*. Therefore, the format

of all manuscripts follows the style required by Comparative Haematology International.

NEUTROPHIL FUNCTION IN IMMATURE ANIMALS: A LITERATURE REVIEW

NEUTROPHIL FUNCTION IN IMMATURE ANIMALS AND MAN

Introduction

According to a study by Martin et al. (1975), the mortality rate for calves from 16 randomly chosen farms averaged 17.3 to 20.2%, and 50% of all deaths occurred during the first week of life. The higher mortality rate of young animals is primarily due to increased susceptibility to a variety of pathogens. Some of these pathogens are not life-threatening in adult animals. Enteric and respiratory infections are major factors causing illness and death in young calves (Bryson et al. 1978; Roy et al. 1980). In humans, 1 to 3 infants per 1000 live births develop life-threatening sepsis and meningitis caused by group B streptococcus (Baker 1979). The increased susceptibility of infants to infection by herpes simplex virus, Listeria monocytogenes, salmonella, Mycobacterium tuberculosis, Toxoplasma gondii and Chlamydia trachomatis also leads to serious disease and high mortality (Wilson 1986).

Reasons for the higher morbidity and mortality observed in immature animals include immaturity of immunologic and nonimmunologic activities (Banks 1982; Wilson 1986). Before developing specific immunity, defense mechanisms of neonatal animals against invading pathogens are mainly dependent on non-specific effector cells (like macrophages and

neutrophils), and humoral factors (like complement components).

Circulating neutrophils are considered an important first line of defense against invading pathogens. Many animal models have been used to evaluate the physiological and biological functions of neutrophils from immature animals as compared to those of neutrophils from adult animals. Increased knowledge of neutrophil function in immature animals will lead to a better understandings of disease development and may lead to improved strategies for prevention and treatment of infection in immature animals.

Neutrophil Adherence and Motility

The migration of circulating neutrophils to the site of infection is an important step in host defense. The whole process requires: 1) recognizing chemotactic factors released by invading microorganisms and from injured tissues, 2) adhering firmly to vascular endothelium via increased expression of adhesion molecules by both neutrophils and endothelial cells in response to inflammatory mediators and chemotactic factors, and 3) reorganizing the cytoskeletal system for facilitating diapedesis and migration to infected tissues.

Defects of neutrophil adherence are considered a cause of increased susceptibility of neonates to bacterial infection.

When using a glass and nylon wool column adherence assay, adherence of neutrophils from normal human newborns (0.18 ± 0.16%) was significantly less than neutrophil adherence in adults (16.0 ± 4.5%). This indicates that decreased neonatal neutrophil adherence to endothelial cells is due to a neutrophil defect (Krause et al. 1982). However, in a study by Zwahlen et al. (1987) using bovine neutrophils, adherence of neonatal neutrophils and adult neutrophils to columns of Sephadex G-25 was comparable in the absence of stimuli and had a similarly increased response when using zymosan-activated plasma (ZAP) (a source of activated complement fragments) as a stimulus. Little is known about neutrophil adherence in immature animals of other species.

Since human neonatal neutrophils have a defective ability to adhere, efforts have been made to investigate Mac-1 (CD11b/CD18) expression on the neutrophil surface. Mac-1, a neutrophil surface glycoprotein, can mediate neutrophil-dependent adherence to endothelial cells. Insufficient induction of surface Mac-1 protein expression by neonatal neutrophils in response to chemotactic factors was demonstrated (Anderson et al. 1987). Furthermore, studies by the same group using human umbilical vein endothelial cell monolayers <u>in vitro</u> indicated that the insufficient induction of surface Mac-1 contributed to diminished transendothelial migration by neonatal neutrophils in response to stimuli (Anderson et al. 1990). Similar results also were reported in

neonatal rabbits (Fortenberry et al. 1992). The molecular basis for decreased induction of Mac-1 surface protein was due to diminished translocation of Mac-1 from the pre-γ fraction (gelatinase-rich granules) after stimulation with formyl-methionyl-leucyl-phenylalanine (FMLP). However, the content of Mac-1 and its distribution within neonatal neutrophils were comparable to those of adult neutrophils (Jones et al. 1990).

Depressed random (chemokinesis) and directed migration (chemotaxis) has been reported in neutrophils from human neonates, infants, and young children (Miller et al. 1971; Klein et al. 1977; Krause et al. 1986), neonatal rats (Schuit et al. 1984), and foals (Coignoul et al. 1984). In contrast, random migration and chemotaxis (using zymosan-activated plasma or C5a) were higher in newborn calves as compared to adult cattle (Zwahlen et al. 1990). In studies of nonmotile neutrophil subpopulations using 31D8 Mab, human neonates had a larger nonmigrating neutrophil subpopulation than did adults. Therefore, it was suggested that the diminished human neonatal neutrophil motility was partially due to increased numbers of nonmotile neutrophils in neonates (Kraus et al. 1989). The same phenomenon has not been studied in other species.

Actin microfilaments are predominant elements in the cytoskeletal system. The dynamic process of actin polymerization is essential to neutrophil motility. Neutrophils from infants showed significantly diminished actin polymerization as compared to neutrophils from adults in

response to FMLP and platelet activating factor (PAF) (Harris et al. 1993). In comparative studies of actin polymerization in bovine neutrophils (Bochsler et al. 1992), there was no significant difference between newborn calves and adult cattle when using opsonized-zymosan as a stimulus. However, adult cattle demonstrated increased and sustained actin polymerization as compared to newborn calves in response to C5a and PAF. This may be due to differences in stimulus-specific responses between newborn calves and mature cattle.

Neutrophil Cytoplasmic Ca^{2+} Flux

Following the activation of neutrophils by stimuli, a rapid rise of intracellular Ca^{2+} concentration acts as an important second messenger (Pozzan et al. 1983). Two sources are thought to contribute to the elevated cytoplasmic Ca^{2+} concentration: a release of Ca^{2+} from intracellular stores and an influx of Ca^{2+} across the plasma membrane. Ca^{2+} flux has been shown to be linked to phagocytosis, actin polymerization, degranulation, superoxide anion generation and chemotaxis (Lew et al. 1984; Lew et al. 1985; Sklar et al. 1985; Sawyer et al. 1985; Di Virgilio et al. 1987).

In the study by Strauss et al. (1985), the uptake of extracellular radioactive calcium (^{45}Ca) by resting neutrophils from neonates was significantly less than those from adults. However, the uptake of ^{45}Ca from extracellular medium was

comparable between neonates and adults following stimulation with sodium fluoride (NaF), FMLP, dimethylsulfoxide and opsonized zymosan particles. In contrast, changes in free intracellular calcium in FMLP-stimulated neonatal neutrophils, as measured by quin2/AM fluorescence, was significantly decreased as compared to FMLP-stimulated adult neutrophils (Sacchi et al. 1984). These results suggest that deficient Ca^{2+} released from intracellular stores may result in some dysfunction of human neonatal neutrophils. Newborn bovine neutrophils can mobilize Ca^{2+} as efficiently as adult bovine neutrophils in response to recombinant human C5a (rhC5a) (Doré et al. 1990).

Neutrophil Cytotoxicity

Antibody dependent cell-mediated cytotoxicity (ADCC) has been proposed to have an important role in antiviral defense (Norrild et al. 1986), tumor immunity (Pollack et al. 1972; Levy et al. 1979), and autoimmune disease (Calder et al. 1973). In ADCC, cytotoxic cells bearing FcR on their surfaces lyse target cells coated with immunoglobulin G (IgG) antibodies (Segal 1990). Among various FcR-bearing cells, the ADCC activity of neutrophils was much more effective and functional at lower antibody levels (Grewal et al. 1977).

The development of ADCC activity have been reported to be required for resistance to some viral infections. Young pigs

had lower ADCC activity against PRV-infected cells as compared to adult pigs (El-Awar et al. 1985). The activity increased to adult levels by 3 months of age (El-Awar et al. 1987). Further studies revealed that the age-dependent deficiency in ADCC was correlated with a lower number of high affinity Fc receptors on neutrophils from young pigs (El-Awar et al. 1991). A study by Hauser et al. (1986) evaluated ADCC activity in 4 age groups of calves: 4 to 5 weeks, 9 to 11 weeks, 16 to 19 weeks, and 12 to 14 months. It was reported that neutrophils from the younger age groups had lower ADCC activity. The reduced FcR expression by bovine neonatal neutrophils (Zwahlen et al, 1992) could contribute to impaired ADCC activity in that age group. In equine neutrophil studies (Coignoul et al. 1984), neutrophils from pony foals (2 to 4 months old) had lower ADCC activity as compared to neutrophils from nonlactating mares (63% vs 70%) but higher as compared to lactating mares (63% vs 54%). The molecular basis for these observations needs to be defined.

Microbicidal Activities

Neutrophils are phagocytic cells which can function in both native immunity and acquired immunity to defend against invading microorganisms (Ross 1992). The pathogens engulfed by neutrophil phagocytosis must be killed by neutrophil microbicidal activities. Microbicidal activities can be

divided into two categories: non-oxidative microbicidal mechanisms and oxidative microbicidal mechanisms.

Substances released from neutrophil cytoplasmic granules are essential for the non-oxidative microbicidal mechanisms. Lysozyme (Elsbach 1980), myeloperoxidase (Klebanoff 1975), elastase (Blondin et al. 1976), alkaline phosphatase (Borgers et al. 1978), lactoferrin (Oram et al. 1968), and cationic proteins (defensins, bactenecins and indolicidin) (Ganz et al. 1985; Romeo et al. 1988; Gennaro et al. 1989; Selsted et al. 1992) have been reported to have important antibacterial activity. Studies comparing granule contents of neutrophils from different species have been reviewed (Styrt et al. 1989; Roth 1994).

The oxidative microbicidal mechanisms are dependent on a series of enzymatic reactions involving activation of an NADPH oxidase, and a multicomponent electron transport system which catalyzes one-electron reduction of oxygen to form superoxide anion. In the acidic environment of the phagocytic vacuole, superoxide anion spontaneously dismutates to form hydrogen peroxide. Myeloperoxidase released from primary granules catalyzes a reaction between hydrogen peroxide and halide ions (iodide, bromide and chloride) resulting in the halogenation of proteins and generation of aldehyde and hypochlorous acid. The myeloperoxidase-hydrogen peroxide-halide system constitutes a potent microbicidal mechanism. Other reactive intermediates formed during this process, including hydroxyl

radical, singlet oxygen, aldehydes and chloramines, also contribute to neutrophil microbicidal activity (Densen et al. 1992).

In studies of the oxidative metabolism of neutrophils from normal term infants, increased activity was reported for some aspects including oxygen consumption (Park et al. 1970), superoxide anion generation when stimulated with opsonized zymosan (Ambruso et al. 1979), hydrogen peroxide production in the resting state (Strauss et al. 1981), hexose monophosphate shunt activity (Park et al. 1970), and nitroblue tetrazolium dye reduction (Humbert et al. 1970; Chandler et al. 1978). In contrast, decreased activity was reported for chemiluminescence (Van Epps et al. 1978; Mills et al. 1979) and hydroxyl radical formation when neonatal neutrophils were stimulated with phorbol myristate acetate (PMA) (Ambruso et al, 1979).

The increased release of hydrogen peroxide was due to decreased levels of glutathione, glutathione peroxidase and catalase in neonatal neutrophils (Strauss et al. 1980). This likely results in neutrophils from neonates having decreased viability. Following stimulation with PMA, the diminished hydroxyl radical generation was suggested to be due to a decreased content of lactoferrin which can catalyze production of hydroxyl radical following stimulation (Ambruso et al. 1984). Additional studies by Strauss et al. (1983) confirmed that neonatal neutrophils generate equivalent to increased

quantities of superoxide anion when stimulated with either opsonized zymosan or PMA as compared to neutrophils from adults. In kinetic studies of superoxide anion generation, neutrophils from neonates were more rapidly activated ($P < 0.02$) than neutrophils from adults. The biochemical basis for these observations was suggested to be a qualitative difference in the fetal oxidase enzyme system (Ambruso et al. 1987).

Studies of superoxide anion generation showed that neonatal bovine neutrophils (< 24 h old) produced less O_2^- (5.7 ± 0.8 nmole $O_2^-/10^6$ cells/5 min, $p < 0.01$) than did adult neutrophils (> 2 yr old) (9.6 ± 2.1 nmol $O_2^-/10^6$ cells/5 min) and fetal neutrophils (210-220 days gestational age) (10.7 ± 0.7 nmol $O_2^-/10^6$ cells/5 min) in response to PMA stimulation. The reduced O_2^- generation ability can persist for at least 7-10 days after birth (Clifford et al. 1989). In kinetic studies, neonatal bovine neutrophils had a significantly ($P < 0.01$) reduced lag time for O_2^- generation (Doré et al. 1991). The impaired respiratory burst activity observed in newborn bovine neutrophils was not due to a differential activity of nor a distinct kinetic of the NADPH oxidase (Doré et al. 1990).

No significant age-related defects were observed in bactericidal activity of neutrophils from foals and piglets (Coignoul et al. 1984; Morris et al. 1987; Hoskinson et al. 1989). In neonatal rats, reduced neutrophil-mediated killing

of Escherichia coli K1 was reported (Lassiter et al. 1988).

Myeloperoxidase is the characteristic constituent of primary granules, and is required for myeloperoxidase-mediated bactericidal mechanisms. Neutrophils obtained from neonates during the first 48 hours after birth contained less myeloperoxidse as compared to adults in both humans and cattle (Rider et al. 1988; Zwahlen et al. 1992). Also, it was reported that neutrophils from premature and newborn rats had only 25% of the myeloperoxidase content found in adults. By 3 weeks of age, the neutrophil myeloperoxidase content reached 50% of the adult concentration (Christensen et al. 1985). Decreased myeloperoxidase content may contribute to decreased microbicidal activity in immature animals. Hauser et al. (1986) reported that there was no significant difference in myeloperoxidase content of neutrophils from young and adult cattle. An increased level of alkaline phosphatase in neonatal neutrophils was observed in newborns from both human and cattle (Rider et al. 1988; Zwahlen et al. 1992).

INFLUENCE OF CYTOKINES ON NEUTROPHIL FUNCTION IN IMMATURE ANIMALS AND MAN

The Influence of Cytokines on Neutrophil Function

Cytokines, regulatory glycoproteins produced by macrophages, endothelial cells, activated T cells (probably activated B cells as well), and stromal cells, are involved in response to inflammation, immunity and hemopoiesis. Through advances in molecular biology, most of the known cytokines have been purified, cloned, and biosynthesized. These recombinant cytokines have been employed as useful tools in studies of immune regulation, cell-cell interaction, and clinical therapy.

Neutrophils play an important role in defense against pathogens. There is growing evidence that cytokines act as potent modulators of various aspects of neutrophil function. The action of several well characterized cytokines on neutrophils is reviewed here.

Colony stimulating factors

Granulocyte/monocyte colony stimulating factor (GM-CSF), a glycoprotein of approximately 22kd molecular weight, was initially defined by it ability to cause proliferation and differentiation of hematopoietic cells.

Lately, the influence of GM-CSF on neutrophil function

has been emphasized. Enhanced neutrophil cytotoxicity toward antibody-coated mammalian cells after treatment with purified GM-CSF was first reported by Lopez et al. (1983). In addition, an important action of GM-CSF on neutrophils is its priming for enhancement of oxidative metabolism (Weisbart et al. 1985), degranulation (Lopez et al. 1986), phagocytosis (Fleischmann et al. 1986; Capsoni et al. 1991) and chemotaxis (Lopez et al. 1986). GM-CSF has no direct effect on the concentration of cytosolic free calcium (Naccahe et al. 1988; Sullivan et al. 1987). Pretreatment of neutrophils with GM-CSF, however, increased the intracellular calcium flux in response to FMLP, PAF, and LTB4 (Naccahe et al. 1988). Increased surface expression of FcRI, FcRIII, CR1, and CR3 on GM-CSF treated neutrophils (Buckle et al. 1989) was suggested to contribute to the enhancement of receptor-mediated neutrophil functions. Gasson et al. (1984) observed that GM-CSF inhibited neutrophil random migration.

Recombinant bovine GM-CSF was also reported to augment antibody-dependent cell-mediated cytotoxicity (ADCC) against bovine herpes virus-infected cells and phagocytosis of Pasteurella multocida and several Staphylococcus spp by neutrophils from dexamethasone-treated and untreated calves (Reddy et al. 1990).

Granulocyte colony stimulating factor (G-CSF) shares similar biological activity with GM-CSF. It enhanced neutrophil phagocytosis, bacterial killing activity,

superoxide anion production, and ADCC activity (Platzer et al. 1985; Lopeze et al. 1983).

Tumor necrosis factors

Tumor necrosis factor (TNF) which caused regression of transplanted tumors in vivo and was cytostatic or cytocidal to some tumor cells in culture was first identified in endotoxin-induced serum (Carswell et al. 1975). Two forms of TNFs have been described: TNF-α (also identified as cachectin) (Beutler et al 1987) and TNF-β (also referred as lymphotoxin) (Newin et al. 1985).

Both TNF-α and TNF-β have been reported to augment phagocytosis and cytotoxicity by neutrophils (Shalaby et al. 1985). Gamble et al.(1985) indicated that increased neutrophil adherence to endothelial cells is partially due to the increased expression of C3bi receptors on TNF-treated neutrophils. After preincubation with recombinant human TNF (rhTNF), neutrophils were primed to have enhanced oxidative burst activity and degranulation in response to FMLP, PMA, and opsonized zymosan (OZ) (Berkow et al. 1987; Ferrante et al. 1988; Ozaki et al. 1988). In addition to the priming role which TNFs play, several pieces of evidence have shown that rhTNF (α and β) alone can induce neutrophil respiratory burst activity, migration, granule secretion (lactoferrin and lysozyme), and increase the F-actin content of neutrophils (Berkow et al. 1987; Figari et al 1987; Klebanoff et al. 1986;

Richter et al. 1989).

Interferons

Interferons are a heterogeneous family of proteins and glycoproteins (IFN-α,β,γ) potentially produced by all nucleated cells in response to virus infection and by some cells after exposure to various other agents.

Enhancement of human and bovine neutrophil ADCC by IFNs (α, β, γ) has been reported (Shalaby et al 1985; Steinbeck et al. 1986). Enhancement of antibody independent neutrophil-mediated cytotoxicity (AINC) by IFN-γ was found only in bovine neutrophils. IFN-γ treatment increased phagocytic activity by neutrophils from human and mouse but not from the bovine (Shalaby et al 1985; Morrison et al. 1987). However, IFN-α augmented phagocytic activity by bovine neutrophils (Ohmann et al. 1984). These data show that the action of IFNs on neutrophil functions varies between species. IFN-α and IFN-γ have neutrophil-migration inhibition activity in both humans and cattle (Bielefeldt et al. 1986; Steinbeck et al. 1989).

Priming of neutrophils for enhanced function after stimulation is dependent on the stimulus used. Pretreatment of human neutrophils with IFNs (α,γ), caused enhanced superoxide anion generation when using FMLP, C5a and NaF as stimuli but not when opsonized zymosan, PMA or A23187 were used (Bielefeldt et al. 1986; Steinbeck et al 1989; Tennenberg et al 1993). The differential priming may be due to

differential involvement in signal transduction pathways initiated by various agents.

Interleukins

Interleukins, a group of proteins produced by macrophages, T cells and bone marrow stromal cells, serve as communication links between leukocytes. They are able to promote cell growth, differentiation, and functional activation (Mizel 1989). So far, at least 14 interleukins have been purified and characterized. Among these, IL-1, IL-2, IL-4, IL-6, and IL-8 have been evaluated for their effects on neutrophil function.

In general, superoxide anion production in response to a number of stimuli can be primed by IL-1, IL-2, IL-4, and IL-6 treatment <u>in vitro</u>. In addition to their priming effect, IL-1, IL-2, IL-4, and IL-6 have been investigated for their direct stimulatory role on the respiratory burst, degranulation, chemotaxis, and calcium mobilization (Ozaki et al, 1987; Kowanko et al 1987; Boey et al. 1989; Borish et al. 1989; Smith et al. 1987). An inhibitory influence of IL-2 on neutrophil random migration was observed whereas IL-1 caused enhanced random migration (Ozaki et al. 1987; Ferrante et al. 1988; Thomsen et al. 1990). Increased phagocytic activity by IL-4 and increased ADCC by IL-6 treated neutrophils were also reported.

IL-8 (also called NAP-1) is produced by various cells

upon stimulation with IL-1 and TNF. It has been found to induce neutrophil shape change, chemotaxis, exocytosis and respiratory burst activity (Baggiolini et al. 1992). The study by Thomsen et al. (1991) pointed out the cross-species activity of recombinant human IL-8 (rhIL-8) on migration and aggregation by canine neutrophils.

The in vivo influence of cytokines on neutrophil function

In addition to in vitro studies, many studies have also been conducted on the in vivo influence of cytokines on neutrophil function. It has been reported that the administration of CSFs, and TNF not only increase neutrophil numbers but also enhance respiratory burst activity (Vadhan et al. 1987; Antman et al. 1988; Steinbeck et al. 1989; Metcalf et al. 1987; Khwaja et al. 1992). Studies with IL-1 showed that there was a significant influx of neutrophils to the site of administration when 5 units of IL-1 were subcutaneously injected. An additive response was observed when both TNF and IL-1 were injected simultaneously (Mason et al. 1989).

Defects in neutrophil phagocytosis and bacterial killing from acquired immunodeficiency syndrome (AIDS) patients were reported to be corrected after GM-CSF infusion (Baldwin et al. 1988). Treatment of chronic granulomatous disease patients with recombinant human IFN-γ (rhIFN-γ) was reported to enhance the respiratory burst and significantly reduce the risk of infection (Ezekowitz et al. 1988; Ezekowitz et al. 1990).

The Influence of Cytokines on Neutrophils from Immature Animals: In vitro and In vivo

As mentioned earlier in this review, immature animals have increased morbidity and mortality from infection. Decreased neutrophil function is thought to contribute to this problem. The use of cytokines to enhance neutrophil function in immature animals and thereby enhance resistance to infection has been investigated.

Cairo et al. (1991) found that rhGM-CSF *in vitro* significantly enhanced C3bi receptor expression on the surface of newborn neutrophils. Additionally, newborn neutrophils primed by rhGM-CSF showed increased adherence and aggregation following either FMLP or A23187 stimulation. Frank et al. (1990) reported that recombinant mouse GM-CSF (rmGM-CSF) which was given intraperitoneally to neonatal rats 6 hours before a 90% lethal challenge dose of *S. aureus*, increased the survival rate of the rats. The defective bactericidal activity of neutrophils from HIV-1 infected children has been shown to be partially corrected by exposure to GM-CSF *in vitro* (Roilides et al. 1990).

Preincubation of human newborn neutrophils with rhIFN-γ can correct their defect in intracellular calcium mobilization and also enhance their chemotactic responses to levels which were not different from adults (Hill et al. 1991). The use of rboIFN-γ in young calves immunosuppressed by dexamethasone

resulted in significantly less pneumonia after Haemophilus somnus challenge (Chiang et al. 1990).

These studies demonstrate the potential for cytokines to enhance neutrophil function in immature animals and to correct the defective function of neutrophils from young patients with HIV infection and immunosupressed young animals. Therefore, enhancement of neutrophil function through cytokine treatment may increase resistance to infection and have a clinical benefit in young animals.

PAPER I.

DIFFERENCES IN NEUTROPHIL FUNCTION IN YOUNG AND MATURE CATTLE AND THEIR RESPONSE TO IFN-γ

ABSTRACT

Neutrophils from young calves (2-4 months old) had significantly lower iodination, oxidative metabolism, chemotaxis and random migration, but higher antibody-independent cell-mediated cytotoxicity (AINC) activity than neutrophils from mature cattle (2-3 years old). Antibody-dependent cell-mediated cytotoxicity (ADCC) and oxidative metabolism were enhanced, and random migration was diminished in both groups by *in vitro* recombinant bovine IFN-γ (rboIFN-γ) treatment. Neutrophils from both groups had enhanced AINC activity and reduced chemotactic responsiveness after incubation in rboIFN-γ, however, these differences were significant only in the mature cattle. RboIFN-γ decreased the cytoplasmic Ca^{2+} flux in both groups. Neutrophils from young calves had lower cytochrome C reduction and iodination activity than mature cattle in response to both phorbol myristate acetate and opsonized zymosan. Lysed neutrophils from young calves had significantly less myeloperoxidase activity than those from mature cattle. It was also observed that the size of neutrophils increased with age in calves.

Key Words: Immunodeficiency; Intracellular calcium; Myeloperoxidase; Oxidative metabolism

INTRODUCTION

Young animals from many species appear to be more susceptible to infectious diseases than adults. This increased susceptibility of young animals to diseases may be due to age-related insufficiency of native or acquired host defense mechanisms (Clifford et al. 1989; Coignoul et al. 1984; El-Awar and Hahn 1991; Hill 1987; Holden et al. 1989; Miller 1979; Mueller et al. 1983; Pross et al. 1977; Quie and Mills 1979; Renshaw et al. 1978). Neutrophils are important in host defense against invading pathogens, especially in young animals that have not yet produced humoral and cell-mediated responses to many pathogens. Several investigations have focused on differences in neutrophil function between young and mature animals. Previous studies reported decreased antibody dependent cell-mediated cytotoxicity (ADCC), iodination and nitroblue tetrazolium (NBT) reduction by neutrophils from young calves as compared to adult cattle (Hauser et al. 1986). Differences have also been observed in O_2^- generation and complement fragment (Cf)-induced membrane shape change between newborn and adult cattle (Clifford et al. 1989; Holden et al. 1989). These observations might help to explain the increased susceptibility of young calves to infectious diseases.

Recombinant interferons have been employed widely in research and shown to be biologically active on various cell

types. Among these, interferon-γ has been reported as a potent modulating factor of neutrophil activation both *in vitro* and *in vivo* in different species (Steinbeck and Roth 1989). Recombinant bovine interferon-gamma (rboIFN-γ) has been shown to enhance the activity of neutrophils from healthy adult cattle *in vitro* and *in vivo* (Ohmann and Babiuk 1986; Roth and Frank 1989; Steinbeck et al. 1986). The influence of rboIFN-γ on neutrophils with suppressed function has also been demonstrated. RboIFN-γ has been shown to enhance the activity of neutrophils which had been suppressed by *in vivo* dexamethasone treatment (Roth and Frank 1989; Steinbeck et al. 1989), exposure to *Brucella abortus* *in vitro* (Canning and Roth 1989), or *in vivo* infection with bovine viral diarrhea virus (Brown et al. 1991). In most cases, the rboIFN-γ had greater activity on neutrophils with suppressed function than on normal neutrophils. The improvement in host defense mechanisms by rboIFN-γ has been shown to be clinically useful. Young calves that were immunosuppressed by dexamethasone administration, then challenged intrabronchially with *Haemophilus somnus* had significantly less pneumonia if they were also given rboIFN-γ (Chiang et al. 1990).

The primary objective of this study was to further define the differences in neutrophil functions between young calves and mature cattle. The second objective was to compare the influence of rboIFN-γ on neutrophil function in young and mature cattle.

MATERIALS AND METHODS

Animals

Twenty four healthy Holstein steer calves (2-4 months old) and ten healthy mature Holstein steers (2-3 years old) were used in the study of neutrophil function. Two additional age groups (five calves of 6 months old and six newborn calves) were compared with the former two age groups in the study of total protein content and cell size of neutrophils.

Neutrophil preparation

Neutrophils were isolated from peripheral blood as previously described (Roth and Kaeberle 1981). Briefly, peripheral blood was collected into acid-citrate-dextrose solution, centrifuged, and the plasma and buffy coat layer discarded. The packed erythrocytes were lysed by brief exposure to hypotonic conditions, and the remaining cells were washed in 0.015M phosphate-buffered saline solution (PBSS;PH 7.2) and suspended in medium 199, containing 25 mM N-[2-Hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid] buffer (HEPES), to a concentration of $1.0x10^8$ cells /ml.

Neutrophil stimulants

Phorbol myristate acetate (PMA) and calcium ionophore (CaI) (Sigma) were each dissolved in dimethyl sulfoxide (DMSO) to a concentration of 2.0 mg/ml and stored at -20°C as a stock

solution. Bovine opsonized zymosan (BOZ) was prepared by incubating zymosan A with fresh bovine serum as described previously (Roth and Kaeberle 1981). All stimulants were diluted in PBSS to the appropriate concentration for use in the assay. The final concentration for each stimulant when incubated with neutrophils was: PMA, 1 μg/ml; CaI, 0.5 μg/ml; BOZ, 1.0 mg/ml.

Treatment of neutrophils with recombinant bovine interferon-γ

Recombinant bovine interferon-γ was provided by Ciba-Geigy, Basel, Switzerland (4.1×10^6 Units/mg). Neutrophils (1.0×10^8 cells/ml) were incubated in rboIFN-γ (1×10^{-8} gm/ml final concentration; diluted in medium 199 with HEPES) for 2 hr at 39°C in a humidified 5% CO_2 atmosphere. Control neutrophil preparations were incubated in medium 199 with HEPES. The neutrophils were then used in the function assays without washing.

Neutrophil function assays

Several parameters of neutrophil function were evaluated as previously described (Roth and Kaeberle 1981; Steinbeck et al. 1986). Briefly, the ability of neutrophils to mediate antibody-independent cell-mediated cytotoxicity (AINC) and antibody-dependent cell-mediated cytotoxicity (ADCC) was evaluated using ^{51}Cr-labeled chicken erythrocytes as target cells with an effector-to-target ratio of 10:1 and a 2-hr

incubation period. Bovine anti-chicken erythrocyte antibodies were used in ADCC, but not for AINC. The activity of the myeloperoxidase-hydrogen peroxide-halide antibacterial system of the neutrophil was evaluated by the iodination reaction with a 20-min incubation period, using opsonized zymosan as the stimulating particle, and ten percent trichloroacetic acid to precipitate the bound iodine. Ingestion of ^{125}I-iododeoxyuridine-labeled Staphylococcus aureus opsonized with anti S.aureus serum was evaluated with a neutrophil to S. aureus ratio of 1:60 and a 10 min incubation time. Lysostaphin was used to remove the extracellular S. aureus. The results were expressed as the percent of the S. aureus ingested. Cytochrome C reduction was determined by means of a microtiter procedure with opsonized zymosan as the stimulant in a 5-min incubation period. The results were expressed as the optical density (550nm-650nm) per 2.5×10^6 PMNs. Random migration was evaluated by measuring the area of random migration under agarose after an 18-hr incubation period. Chemotaxis was evaluated in an under agarose assay after a 2-hr incubation period. Zymosan activated bovine serum was used as the chemoattractant. The chemotactic index was calculated by dividing the distance of migration toward the chemotactic factor by the distance of migration toward the control medium.

Evaluation of myeloperoxidase activity

The relative myeloperoxidase activity of neutrophil lysates was determined using a modification of the iodination assay. One ml of standardized neutrophils ($5x10^7$ cells/ml) was centrifuged at 500x g for 10 mins at room temperature. The supernatant was discarded and the packed neutrophils were lysed by addition of 1 ml distilled H_2O for 20 min. Twenty five μl of neutrophil lysate was mixed with an equal volume of Hanks' balanced salt solution (HBSS) without phenol red. The standard reaction mixture contained 50 μl of 40 nmole NaI, 50 μl of 0.05 μCi ^{125}I (carrier free in 0.1 M NaOH, New England Nuclear, Boston, MA, USA), 50 μl neutrophil lysate mixture, and 50 μl of 1 mM H_2O_2 in a total volume of 0.5 ml Earle's balanced salt solution (EBSS). After 5 min incubation at 39°C, ten percent trichloroacetic acid was added to precipitate the bound iodine.

Kinetic assay of neutrophil function

Oxidant production and cytoplasmic calcium flux were measured using a SLM 8000C photon-counting spectrofluorometer (SLM Instruments, Inc. Urbana, IL). Opsonized zymosan was used as a stimulant at a final concentration of 1.0 mg/ml. Oxidant production assay: The oxidant production assay was a modification of a procedure which indirectly measures O_2^- production by neutrophils as previously described (Brown and Roth 1991; Hyslop and Sklar 1984). Briefly, neutrophils

produced O_2^- after exposure to appropriate stimulants. The O_2^- was rapidly converted into H_2O_2 in the presence of superoxide dismutase. The H_2O_2 oxidized p-hydroxyphenylacetate (PHPA) to a fluorescent product 2,2'-dihydroxy-biphenyl-5,5'-diacetate $(PHPA)_2$ in the presence of horseradish peroxidase. This fluorescent product emitted light at a 400-nm wavelength when excited by light at 340-nm wavelength. Each cuvette prewarmed to 39°C contained 2.5 ml of HBSS without phenol red, 100 µl neutrophils ($5x10^7$ cells/ml), and 75 µl of a cocktail reagent consisting of a ratio of 10:10:25 of superoxide dismutase (8.0 mg/ml of PBSS), horseradish peroxidase (8.0 mg/ml of PBSS), and PHPA (10.0 mg/ml of PBSS). The appropriate stimulant was added 20 seconds after initiation of the assay. Fluorescence readings at 400 nm were taken every 3 seconds for 300 seconds including a base line reading at time 0.

Cytoplasmic calcium flux: Fura-2/AM (Calbiochem-Behring Diagnostics, San Diego, CA) was used to measure cytoplasmic calcium flux as described previously (Brown and Roth 1991; Grynkiewicz et al. 1985). Briefly, $5x10^7$ neutrophils were loaded with Fura-2/AM by incubating PMNs in 10 µM Fura-2/Am at 39°C for 30 minutes. The cuvette containing 2.5 ml of HBSS without phenol red and 100 µl neutrophils ($5x10^7$ cells/ml) loaded with Fura-2 was prewarmed to 39°C. The fluorescence ratios were recorded by measuring the light emission at 510-nm when alternating the excitation wavelength between 340-nm and 380-nm. Fura-2 shifted its peak absorbance from 380-nm to

340-nm upon binding calcium. The fluorescence ratio reading was taken every 2 seconds for 100 seconds. Opsonized zymosan was added 10 seconds after the beginning of the assay.

Measurement of total neutrophil protein

Neutrophils from 4 age groups had been stored at -70°C at a concentration of $5x10^7$ cells/ml in PBSS until the assay was to be conducted. The cell suspension was diluted to $6.25x10^6$ cell/ml after one cycle of freeze-thawing and sonicated for 20s in an ice bath. Total protein content of the cell lysates were determined using the Bio-Rad (Richmond, CA) protein reagent with bovine gamma globulin as a standard.

Determination of relative cell size

The relative size of neutrophils from 4 age groups of cattle was measured using a flow cytometer (Epics 752, Coulter Electronics, Inc. Hialeah, Florida). Data from a total of 10,000 cells from each neutrophil preparation were collected in a Forward Angle Light Scatter (FALS) histogram gated on FALS and 90° light scatter using a wavelength of 488 nm with a power setting of 250 mW. A dichroic filter and a 10% neutral density filter were used for the 90° light scatter and forward angle light scatter detectors respectively. Latex beads (Polysciences, Warrington, PA) with a diameter of 6.49 microns ± 0.5 microns and fixed neutrophils were used as standard references for instrument alignment, and were set to a

specific mean channel position prior to the analysis of the nonfixed neutrophils.

Statistical analysis

Mean and SEM were calculated for each neutrophil function value for each treatment group. An analysis of variance was used to determine the level of significance of the differences between the age groups and the differences between control and rboIFN-γ treated-cells in the same age group. The date of the assay was used as a blocking factor for all assays. In the kinetic neutrophil assays, data from selected time points were compared between groups.

RESULTS

Comparison of neutrophil function between young and mature animal groups: The young calves had significantly lower iodination ($p<0.01$), cytochrome C reduction ($p<0.01$), chemotaxis ($p<0.05$) and random migration ($p<0.01$) as compared to mature cattle. The antibody-independent cell-mediated cytotoxicity (AINC) of neutrophils from young calves was higher than that of neutrophils from the mature cattle ($p<0.01$). There was no significant difference in S. aureus ingestion or ADCC between neutrophils from young and mature cattle (Fig 1). The intracellular calcium mobilization in response to BOZ stimulation was not significantly different between the two age groups (Fig 2). Oxidant production was significantly ($p<0.01$) lower in neutrophils from young calves as compared to neutrophils from mature cattle (Fig 3).

The effect of rboIFN-γ on neutrophil function: In both age groups, neutrophil cytochrome C reduction, ADCC, and oxidant production were significantly enhanced, and random migration was inhibited by rboIFN-γ treatment in vitro. The rboIFN-γ had no significant influence on iodination or S. aureus ingestion in either age group (Figs 1,3). Treatment with rboIFN-γ reduced the increase in intracellular Ca^{2+} concentration in response to BOZ in both age groups ($p<0.05$)

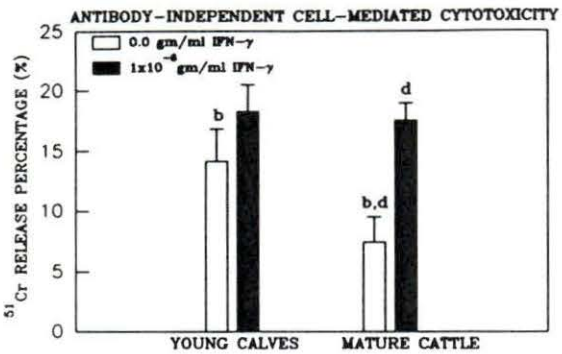
(Fig 2). AINC activity was increased and chemotaxis was inhibited in neutrophils from both age groups treated with rboIFN-γ, however, the differences were only significant ($p<0.01$) in the older group (Fig 1).

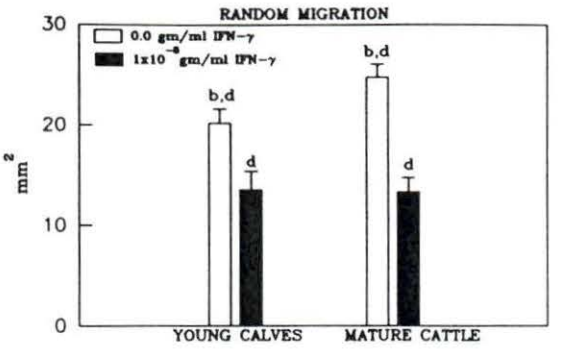
The effect of PMA and CaI on neutrophil function: When the neutrophil preparations from both groups were stimulated by PMA, or PMA plus CaI, those from the young animal group had significantly lower iodination activity and cytochrome C reduction than did those from the mature animal group ($p<0.01$) (Fig.4). Addition of CaI alone did not stimulate neutrophil iodination or cytochrome C reduction.

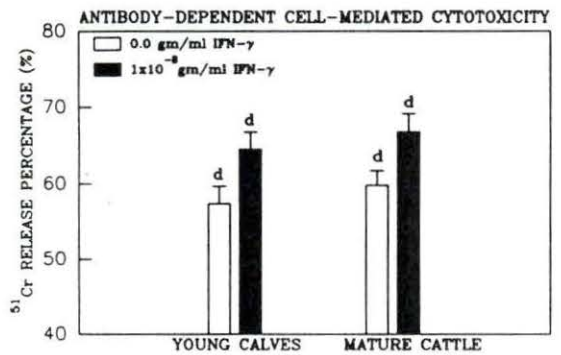
Activity of myeloperoxidase: Neutrophil lysates from young calves had significantly ($p<0.05$) less myeloperoxidase activity as compared to neutrophil lysates from mature cattle (Fig 5).

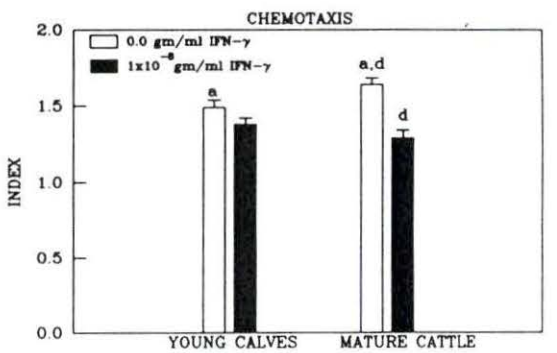
Total protein content and cell size: The total protein content and relative cell size of neutrophils from 4 different age groups of cattle were compared. Neutrophils from newborn calves and adult cattle had a higher total protein content compared to neutrophils from calves 2-4 months of age and 6 months of age. However, the only statistically significant difference occurred between the group that was 2-4 months old and the group of newborn calves ($p<0.05$). Relative neutrophil size was found to significantly increase with age change ($p<0.05$) (Table 1).

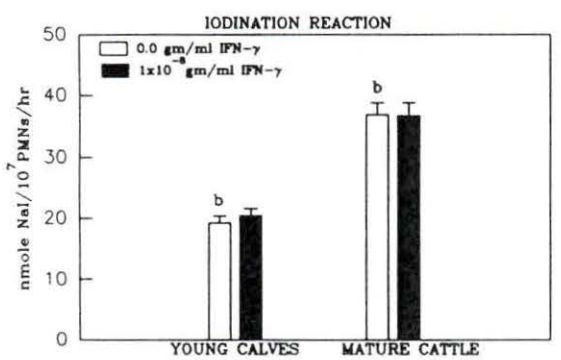
Fig.1 Functional comparisons of neutrophils from young and mature cattle with and without <u>in vitro</u> rboIFN-γ treatment. Data are expressed as mean ± SEM (n=30 to 37). $^{a}p<0.05$, $^{b}p<0.01$: significant difference between the two age groups; $^{c}p<0.05$, $^{d}p<0.01$: significant difference between neutrophils with and without rboIFN-γ treatment in the same age group.

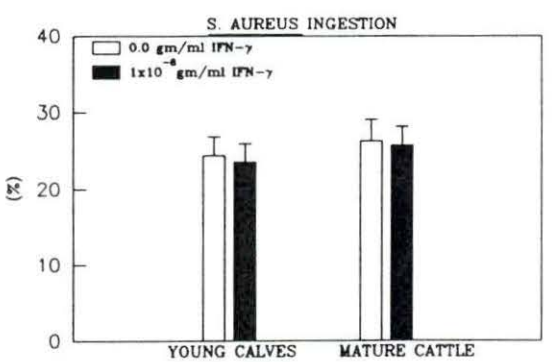

ANTIBODY-INDEPENDENT CELL-MEDIATED CYTOTOXICITY
0.0 gm/ml IFN-γ
1x10⁻⁸ gm/ml IFN-γ
51Cr RELEASE PERCENTAGE (%)
25
20
15
10
5
0
b
d
b,d
d
YOUNG CALVES
MATURE CATTLE


RANDOM MIGRATION
0.0 gm/ml IFN-γ
1x10⁻⁸ gm/ml IFN-γ
mm2
30
20
10
0
b,d
d
b,d
d
YOUNG CALVES
MATURE CATTLE


ANTIBODY-DEPENDENT CELL-MEDIATED CYTOTOXICITY
0.0 gm/ml IFN-γ
1x10⁻⁸ gm/ml IFN-γ
51Cr RELEASE PERCENTAGE (%)
80
70
60
50
40
d
d
d
d
YOUNG CALVES
MATURE CATTLE


CHEMOTAXIS
0.0 gm/ml IFN-γ
1x10⁻⁸ gm/ml IFN-γ
INDEX
2.0
1.5
1.0
0.5
0.0
a
a,d
d
YOUNG CALVES
MATURE CATTLE


IODINATION REACTION
0.0 gm/ml IFN-γ
1x10⁻⁸ gm/ml IFN-γ
nmole NaI/10⁷ PMNs/hr
50
40
30
20
10
0
b
b
YOUNG CALVES
MATURE CATTLE


S. AUREUS INGESTION
0.0 gm/ml IFN-γ
1x10⁻⁸ gm/ml IFN-γ
(%)
40
30
20
10
0
YOUNG CALVES
MATURE CATTLE

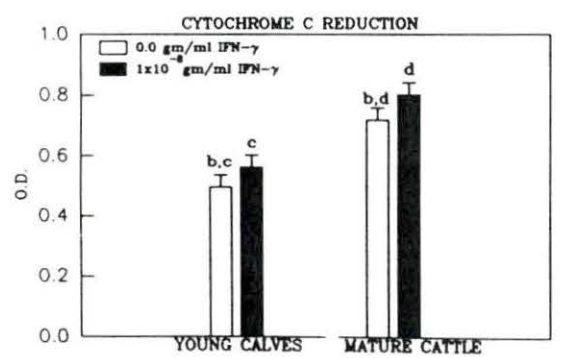

CYTOCHROME C REDUCTION
0.0 gm/ml IFN-γ
1x10⁻⁸ gm/ml IFN-γ
O.D.
1.0
0.8
0.6
0.4
0.2
0.0
b,c
c
b,d
d
YOUNG CALVES
MATURE CATTLE

Fig.2 Kinetics of the cytoplasmic calcium flux by bovine neutrophils with and without <u>in vitro</u> rboIFN-γ treatment in young and mature cattle. There was no significant ($p>0.05$) difference in intracellular Ca^{2+} mobilization between the two age groups (○-○ vs □-□) in response to bovine opsonized zymosan (BOZ) stimulation added at 10 sec. The rise in intracellular Ca^{2+} concentration of neutrophils in response to BOZ stimulation was significantly ($p<0.05$) reduced by rboIFN-γ treatment in both age groups (○-○ vs ●-●; □-□ vs ■-■). Data are expressed as mean values (n=6 to 11). SEM of each time point does not exceed 19% of the mean.

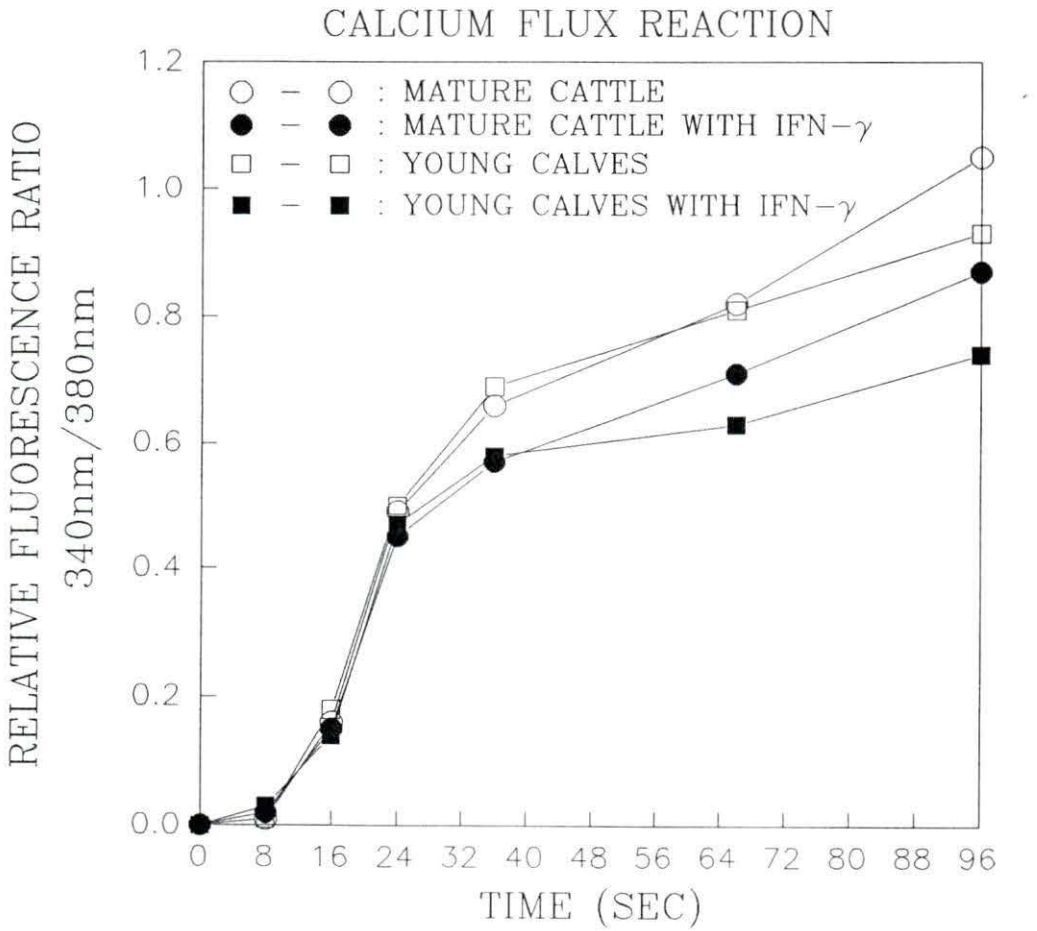

CALCIUM FLUX REACTION
RELATIVE FLUORESCENCE RATIO
340nm/380nm
○ – ○ : MATURE CATTLE
● – ● : MATURE CATTLE WITH IFN–γ
□ – □ : YOUNG CALVES
■ – ■ : YOUNG CALVES WITH IFN–γ
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0 8 16 24 32 40 48 56 64 72 80 88 96
TIME (SEC)

Fig.3 Kinetics of oxidant production by bovine neutrophils with and without <u>in vitro</u> rboIFN-γ treatment in young and mature cattle. Neutrophils from mature cattle had significantly ($p<0.01$) higher O_2^- generation than those from young calves (○-○ vs □-□). RboIFN-γ treatment enhanced O_2^- generation of neutrophils from both age groups in response to bovine opsonized zymosan stimulation added at 20 sec ($p<0.01$) (○-○ vs ●-●; □-□ vs ■-■). Data are expressed as mean values (n=17). SEM of each time point does not exceed 5% of the mean.

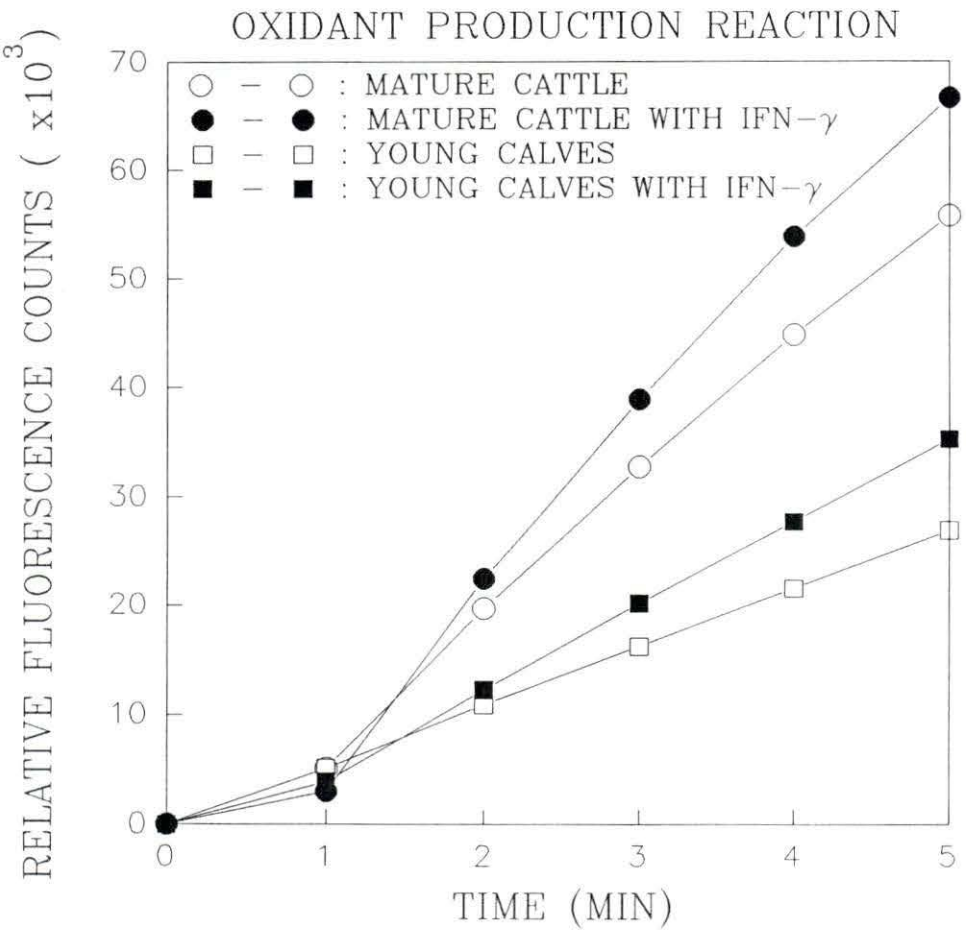
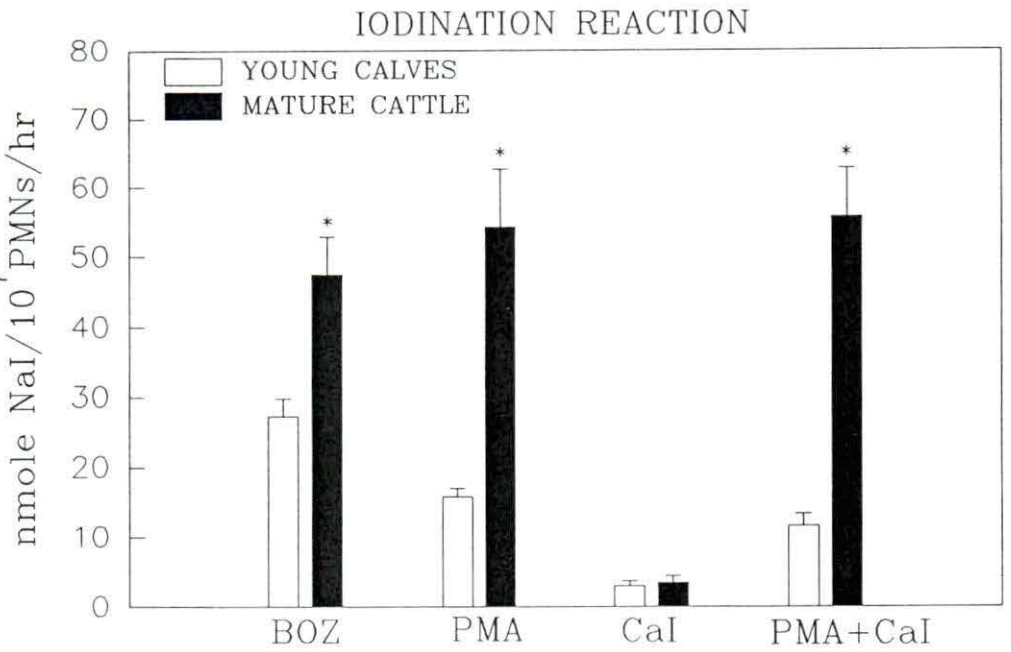

OXIDANT PRODUCTION REACTION
RELATIVE FLUORESCENCE COUNTS (x10^3)
TIME (MIN)
○ — ○ : MATURE CATTLE
● — ● : MATURE CATTLE WITH IFN-γ
□ — □ : YOUNG CALVES
■ — ■ : YOUNG CALVES WITH IFN-γ
0
10
20
30
40
50
60
70
0
1
2
3
4
5

Fig.4 Iodination reaction and cytochrome c reduction of bovine neutrophils in response to bovine opsonized zymosan (BOZ), phorbol myristate acetate (PMA), calcium ionophore (CaI), and PMA+CaI in young and mature cattle. Data are expressed as mean ± SEM (n=11). $^{*}p<0.01$.


IODINATION REACTION
YOUNG CALVES
MATURE CATTLE
nmole NaI/10^7 PMNs/hr
80
70
60
50
40
30
20
10
0
*
BOZ
PMA
CaI
PMA+CaI

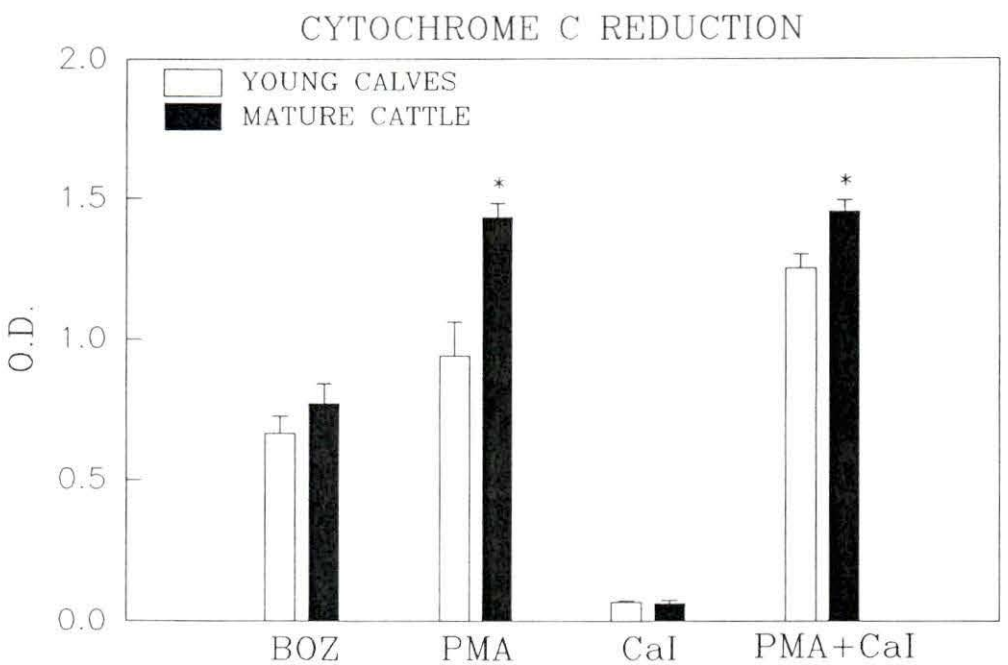

CYTOCHROME C REDUCTION
YOUNG CALVES
MATURE CATTLE
O.D.
2.0
1.5
1.0
0.5
0.0
*
BOZ
PMA
CaI
PMA+CaI

Fig.5 Myeloperoxidase activity of lysates of neutrophils from young and mature cattle. Data are expressed as mean ± SEM (n=6). $^{*}p<0.05$.

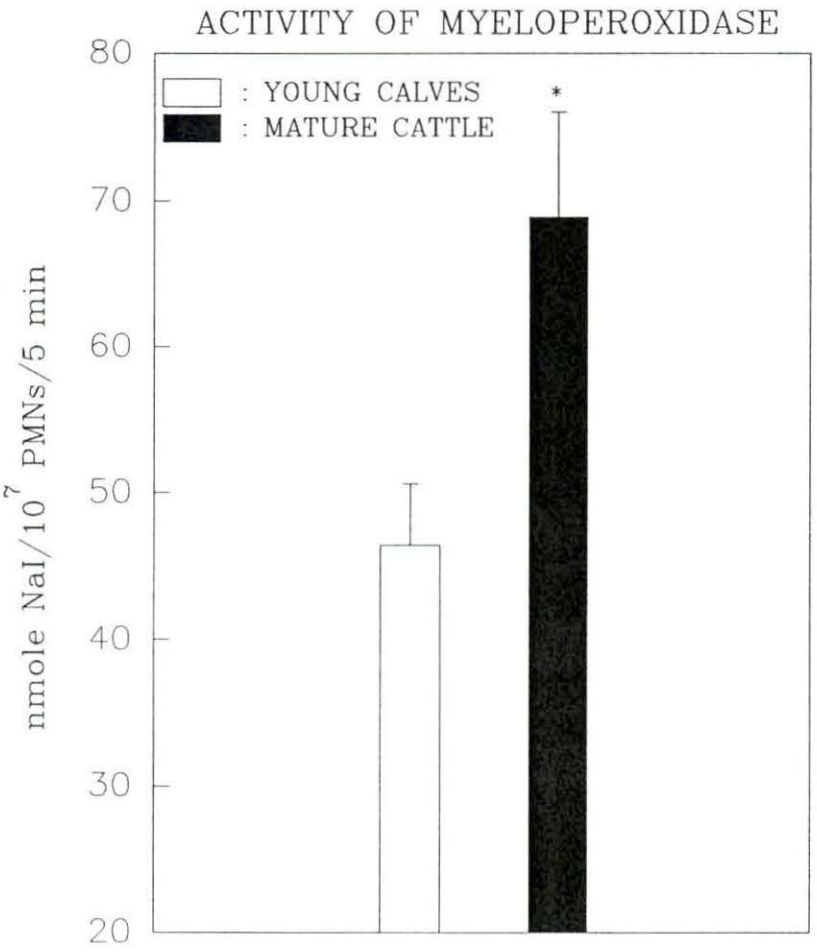

ACTIVITY OF MYELOPEROXIDASE
: YOUNG CALVES
: MATURE CATTLE
*
nmole NaI/10^7 PMNs/5 min
80
70
60
50
40
30
20

Table 1

Comparisons of Total Protein Content and Relative Size of PMNs Among 4 Age Groups of cattle

	Groups			
	2-3 Yr	6 Mo	2-4 Mo	Newborn
Total Protein *	640 ± 32 (n = 5)	578 ± 32 (n = 5)	572 ± 24[a] (n = 9)	662 ± 32[a] (n = 5)
Relative Size **	128 ± 2[a] (n = 5)	125 ± 2[b] (n = 5)	122 ± 2[a] (n = 10)	113 ± 2[a,b] (n = 6)

Values expressed as mean ± SEM.
Values with the same superscript are significantly different ($p<0.05$).
* $\mu g/6.25 \times 10^6$ cell lysate.
** Forward angle light scatter channel unit mean.

DISCUSSION

Neutrophils from the young calves had decreased oxidative metabolism and iodination, two activities which are important for the killing of bacteria by neutrophils. Chemotaxis and random migration of neutrophils were also decreased in young calves as compared to mature cattle. Treatment with rboIFN-γ *in vitro* altered the functions of neutrophils from young calves similarly to those from mature cattle. In both age groups, cytochrome C reduction, oxidant production, and antibody-dependent cell-mediated cytotoxicity (ADCC) were significantly enhanced and random migration and Ca^{2+} mobilization were significantly inhibited by rboIFN-γ. *Staphylococcus aureus* ingestion and iodination were not influenced by rboIFN-γ treatment in either group. The observed changes in neutrophil functions in young calves due to rboIFN-γ may help to explain the increased resistance to *Haemophilus somnus* pneumonia observed in young calves treated with rboIFN-γ (Chiang et al. 1990).

In this study, neutrophils from young calves (2-4 mo) had lower ADCC activity as compared to neutrophils from mature cattle. This may be related to the decreased size of neutrophils reported here and/or to reduced expression of Fc receptors observed on neutrophils from bovine neonates (Zwahlen et al. 1992). RboIFN-γ significantly enhanced ADCC activity in both age groups. This increased activity might be

due to an induction of high affinity Fc receptors after incubation with IFN-γ which has been observed in human neutrophils (Perussia et al. 1987).

The AINC activity was higher in neutrophils from the young group than in neutrophils from the mature group. It has been shown that the enhancement of AINC by rboIFN-γ depends upon RNA transcription, protein synthesis, and synthesis of arachidonic acid metabolites by neutrophils from adult cattle (Steinbeck et al 1989). Although the AINC activity was increased by rboIFN-γ treatment in both age groups, the difference was not significant in the young calves. The lack of statistical significance may be due to the higher level of AINC activity present in the young calf neutrophils before rboIFN-γ treatment.

The ability of the neutrophil to respond to chemotactic stimuli and arrive at a site of inflammation involves cooperation among the microtubular system, microfilament system, actin polymerization, Ca^{2+} mobilization, and expression of surface adherence proteins (Hallett 1989; Hill 1987; Sawyer et al. 1985). Several reports (Hill 1987; Miller 1979; Quie and Mills 1979) have shown that neutrophils from human neonates are deficient in their ability to move toward chemotactic stimuli. Similar impaired motility was also observed in foals (Coignoul et al. 1984). Results reported here indicate that neutrophils from 2-4 month old calves also have lower activity in both chemotaxis and random migration

than those from mature cattle.

A rapid increase in intracellular Ca^{2+} concentration is an essential step in the signal transduction pathway that leads to a cellular response after opsonized zymosan binds to a receptor on the surface of the neutrophil. One possibility to explain the different activities of neutrophils from young and mature cattle could be an altered intracellular Ca^{2+} flux in the young calves. Therefore the change in intracellular Ca^{2+} concentration after BOZ stimulation was examined in this study. No significant difference was found between the two groups (Fig 2). A previous study also reported comparable Ca^{2+} mobilization in response to recombinant human C5a between newborn bovine neutrophils and adult bovine neutrophils (Doré et al. 1990). Therefore the change in intracellular Ca^{2+} concentration after stimulation seems to be comparable between young calves and mature cattle and is probably not responsible for the lower function in neutrophils from young calves.

The reduction of random migration by rboIFN-γ treatment in both groups (Fig 1) was consistent with previous studies indicating that rboIFN-γ is a migration inhibition factor (Steinbeck et al. 1986; Steinbeck et al. 1989).

In the present study young calves had decreased O_2^- generation as compared to mature cattle (Figs 1,3). Previous studies using human neonatal blood and cord blood reported increased O_2^- and higher activity of NADPH oxidase in neonatal neutrophils and fetal neutrophils (Ambruso et al. 1987;

Strauss and Snyder 1983). Neutrophils from newborn calves had decreased O_2^- generation when PMA was used as a stimulus, but they had increased O_2^- generation when OZ was used as a stimulus (Clifford et al. 1989; Doré et al. 1991; Holden et al. 1989). It was suggested that there was a stimulus-specific deficit in O_2^- generation in the newborn calf. Our results indicate that by a few weeks of age the ability of neutrophils from young calves to generate O_2^- is decreased when either OZ or PMA is used as a stimulus (Figs 1,3,4).

Myeloperoxidase deficiency has been observed in neonatal human neutrophils (Rider et al. 1988). Similarly, the present study showed deficient activity of myeloperoxidase in young calves (2-4 Mo). This, along with the decreased oxidative burst, is probably responsible for the impaired activity of the myeloperoxidase-hydrogen peroxide-halide system (iodination). It also indicates that the myeloperoxidase deficiency exists at least for four months after birth in developing calves. The total protein content of neutrophils from newborn calves was similar to the total protein content of neutrophils from adult cattle, even though the neutrophils from adult cattle were larger (Table 1). This indicates that there is a greater concentration of protein in the newborn calf neutrophils than in neutrophils from adult cattle. The neutrophil total protein decreased significantly by 2-4 months of age. The basis for the changes in neutrophil total protein with age is not known.

Binding of opsonized zymosan to receptors on the neutrophil membrane would be expected to activate protein kinase C (PKC) via a signal transduction pathway (Omann et al. 1987; Roos et al. 1981). Activated-PKC can subsequently phosphorylate the NADPH-oxidase to initiate the respiratory burst (Cooke and Hallett 1985; Hallett 1989; Tauber 1987). Phorbol myristate acetate (PMA) by-passes much of the signal transduction pathway and directly activates PKC (Brown and Roth 1991; Doré et al. 1991; Nishihira and O'flaherty 1985). Calcium ionophore has been shown to cause redistribution of Ca^{2+} from intracellular stores and influx of Ca^{2+} from the extracellular medium (Hallett 1989). This study demonstrated that both BOZ-stimulated and PMA-stimulated calf neutrophils had decreased cytochrome C reduction and an impaired activity of the myeloperoxidase-hydrogen peroxide-halide system as compared to neutrophils from mature cattle (Fig 4). Calcium ionophore alone, however, did not induce cytochrome C reduction or iodination (Fig 4). This indicated that without PMA-mediated or diacylglyceride-mediated activation of PKC, the increase in cytoplasmic Ca^{2+} concentration alone cannot initiate the respiratory burst in bovine neutrophils. In addition, the flux of Ca^{2+} in response to BOZ stimulus was similar in both age groups (Fig 2). Therefore, deficient cytoplasmic Ca^{2+} mobilization in young calves was not a potential explanation for decreased oxidative metabolism and iodination activities. Other factors involved in the

generation of O_2^- such as deficient metabolism of membrane-phospholipids after stimulation, decreased amount and/or altered activation of membrane-associated NADPH oxidase, decreased activity of the hexose monophosphate shunt which provides the NADPH for the oxidase enzyme, or the presence of inhibitors in young calves may be responsible for the decreased oxidative metabolism.

RboIFN-γ enhanced cytochrome C reduction and the kinetic assay of oxidant production in both animal groups, but did not influence the iodination assay (Figs 1,3) in either group. This suggested that oxidative metabolism is not the limiting factor in the iodination activity. Intracellular Ca^{2+} flux was reduced after rboIFN-γ treatment in both animal groups (Fig 2). *In vitro* treatment with rboIFN-γ has previously been shown to diminish intracellular Ca^{2+} fluxes in neutrophils from normal mature cattle, and to enhance the Ca^{2+} flux in neutrophils which had a defective Ca^{2+} flux due to *in vivo* infection with bovine viral diarrhea virus (Brown et al. 1991).

In conclusion, several aspects of neutrophil function were decreased in young calves as compared to mature cattle. This may help to explain why young calves are more susceptible to infectious diseases. RboIFN-γ, a potentially useful immunomodulator, had a similar *in vitro* influence on the neutrophils from both age groups, indicating that neutrophils from young calves are capable of responding to this cytokine

in a manner similar to neutrophils from adult cattle.

Acknowledgement. This work was partially supported by a grant from the Iowa Livestock Health Advisory Council. The authors wish to thank Dr. Kristi R. Harkins and Mrs. Padmasree Chigurupati for assistance in the analysis of cell size by flow cytometer.

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PAPER II.

EFFECT OF GENISTEIN, A TYROSINE KINASE INHIBITOR, ON BOVINE NEUTROPHIL FUNCTION

ABSTRACT

Genistein, a specific inhibitor for tyrosine kinases, was used to investigate the possible role of tyrosine kinases in bovine neutrophils. After a 3 hour pre-incubation of bovine neutrophils with genistein (60 ug/ml), antibody independent cell-mediated cytotoxicity (AINC), and antibody dependent cell-mediated cytotoxicity (ADCC) were significantly decreased. Random migration, opsonized zymosan-induced iodination and opsonized zymosan-induced superoxide anion production were inhibited in a dose dependent manner (0-60 ug/ml) by genistein. Superoxide anion production, however, was not inhibited by genistein (60 ug/ml) when using phorbol myristate acetate as a stimulus. This indicates that genistein does not inhibit protein kinase C (PKC)-dependent NADPH oxidase activity when receptor-mediated initiation is bypassed. Apparently, tyrosine kinases are not needed for signal transduction in the generation of superoxide anion after PKC activation. Tyrosine kinase(s) may be involved in early stages of signal transduction in a PKC-dependent pathway or it may participate in a PKC-independent pathway in bovine neutrophils when antibody or C3b coated particles are used as a stimulus. The results presented here suggest that a tyrosine kinase(s) is involved in various functions of bovine neutrophils.

INTRODUCTION

Dysfunction of neutrophils may be due to stress (Kelly et al. 1988), viral infection (Briggs et al. 1988; Brown et al. 1991),bacterial toxins (O'Brien et al. 1987; Czuprynski et al. 1989; Kagonyera et al. 1989; Paulsen et al. 1990; Hoien-Dalen et al. 1990; Thomas et al. 1991) or inadequate nutrition (Jones et al. 1981; Aziz et al. 1986; MacPherson et al. 1987; Olkowski et al. 1990). Also, it has been observed that neutrophil function is decreased in young calves as compared to mature cattle (Hauser et al. 1986; Clifford et al. 1989; Doré et al.1991; Lee et al. 1992). The suppression of neutrophil function is associated with increased susceptibility to infectious disease (Roth et al. 1982; Nagahata et al. 1987; Roth et al. 1989; Chiang et al. 1990; Kehrli et al. 1992). Preventing suppression of neutrophil function should increase the animals' resistance to infection. Understanding the molecular basis for dysfunction is of importance for designing methods to improve neutrophil function.

Neutrophils can be activated by immune complexes to initiate the respiratory burst, release granule contents and conduct cell-mediated cytotoxicity (Densen et al. 1992). Spontaneous movement is another important physiological function in neutrophils. The biochemical events and/or

pathways involved in mediating bovine neutrophil function are not completely understood.

The phosphorylation of proteins on tyrosine residues by protein tyrosine kinases (PTKs) has been shown to be an important mechanism for the regulation of cellular activities in numerous cell systems. Tyrosine phosphorylation of membrane associated proteins by stimulation with N-formyl-methionyl-leucyl-phenylalanine (FMLP) was observed in rabbit and human neutrophils (Huang et al. 1988; Gomez-Cambronero et al. 1989; Berkow et al. 1990). In studies by Berkow et al. in 1989, it was first demonstrated that the particulate fraction containing tyrosine kinase activity which had a molecular mass of 50-60 Kd was inhibited by a novel tyrosine kinase inhibitor ST638. Superoxide anion (O_2^-) generation induced by FMLP, opsonized zymosan (OZ), and sodium fluoride (NaF) in intact neutrophils was inhibited by ST638 as well. Therefore, tyrosine kinase activity has been shown to correlate with O_2^- generation in response to stimulation with agonists in human neutrophils (Berkow et al. 1989). Also, the expression of $p55^{c\text{-}fgr}$ with tyrosine kinase activity was found in normal human neutrophils (Gutkind et al. 1989).

Erbstatin, another novel tyrosine kinase inhibitor, has been shown to inhibit the production of O_2^- induced by FMLP and platelet-activating factor (Naccache et al. 1990). In addition, both spontaneous and directed migration of human

neutrophils induced by FMLP and leukotriene B_4 were diminished by Erbstatin (Gaudry et al. 1992).

Genistein [5,7-dihydroxy-3-(4-hydroxyphenyl)-4H-1-benzopyran-4-one; 4',5,7-trihydroxyisoflavone], is an isoflavone compound, isolated from fermentation broth of Pseudomonas sp that has been reported to inhibit protein tyrosine kinase activity (Ogawara et al. 1986; Akiyama et al. 1987). It also was shown to have oestrogenic activity (Brodbury et al. 1951) and to act as a β-galactosidase inhibitor (Hazato et al. 1979). The concentrations of genistein that inhibited 50% of tyrosine kinase activity of the epithelial growth factor (EGF) receptor, $pp60^{v\text{-}src}$ and $pp110^{gag\text{-}fes}$ were 6-8 ug/ml in an in vitro system. In contrast, there was no inhibitory effect of genistein on the activity of cAMP-dependent protein kinase even at a concentration of 100 ug/ml. The activity of protein kinase C was inhibited 40% by 100 ug/ml of genistein in a purified enzyme system (Akiyama et al. 1987; Akiyama et al. 1991). The specificity of genistein as an inhibitor of tyrosine kinases has allowed it to be used as an important tool for investigating the role of tyrosine phosphorylation in various cells.

It has recently been shown that O_2^- production by human neutrophils stimulated with FMLP, soluble aggregated human IgG, tumor necrosis factor-α (TNF-α) and anti-integrin (CD18 and CD11a) antibodies, was inhibited by genistein (0.5-50 ug/ml) (Kusunoki et al. 1992; Laudanna et al. 1993). Also,

genistein was used to demonstrate the role for tyrosine kinases in enhancement of FMLP-induced generation of O_2^- by human neutrophils which were primed with granulocyte-colony stimulate factor (G-CSF) and TNF-α (Akimaru et al. 1992; Tanimura et al. 1992). Genistein at a concentration of 30 uM (8 ug/ml) blocked the spreading of neutrophils and it also inhibited hydrogen peroxide generation by human neutrophils exposed to TNF in a dose dependent manner (Fuortes et al. 1993).

The purpose of the study presented here was to determine the possible role of protein tyrosine kinases in bovine neutrophil function by using genistein, a tyrosine kinase inhibitor.

MATERIALS AND METHODS

Animals

Ten healthy Holstein steers (2-3 years old) were used as a source of neutrophils.

Neutrophil preparation

Neutrophils were isolated from peripheral blood as previously described (Roth et al. 1981). Briefly, peripheral blood was collected into acid-citrate-dextrose solution, centrifuged, and the plasma and buffy coat layer discarded. The packed erythrocytes were lysed by brief exposure to hypotonic conditions, and the remaining cells were washed in 0.015M phosphate-buffered saline solution (PBSS;PH 7.2) and suspended in medium 199, containing 25 mM N-[2-Hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid] buffer (HEPES), to a concentration of 1.0×10^8 cells/ml.

Neutrophil stimulants

Phorbol myristate acetate (PMA) was dissolved in dimethyl sulfoxide (DMSO) to a concentration of 2.0 mg/ml and stored at -20°C as a stock solution. Bovine opsonized zymosan (BOZ) was prepared by incubating zymosan A with fresh bovine serum as described previously (Roth et al. 1981). All stimulants were diluted in PBSS to the appropriate concentration for use in the assay. The final concentration for each stimulant when

incubated with neutrophils was: PMA, 1 μg/ml; BOZ, 1.0 mg/ml.

Treatment of neutrophils with genistein

Genistein (GIBCO; Gaithersburg, MD) was dissolved in dimethyl sulfoxide (DMSO) to a concentration of 10 mg/ml and stored at -20°C as a stock solution. Neutrophils (1.0×10^8 cells/ml) were incubated in vitro with an equal volume of varying concentrations of genistein for 3 h at 39°c in a humidified 5% CO_2 atmosphere. Neutrophils were incubated in DMSO (0.1%) as a control. The neutrophils were then used in the function assays without washing.

Neutrophil function assays

Several parameters of neutrophil function were evaluated as previously described (Roth et al. 1981; Steinbeck et al. 1986). Briefly, the ability of neutrophils to mediate antibody-independent cell-mediated cytotoxicity (AINC) and antibody-dependent cell-mediated cytotoxicity (ADCC) was evaluated using ^{51}Cr-labeled chicken erythrocytes (cRBCs) as target cells with an effector-to-target ratio of 10:1 and a 2-hr incubation period. Bovine anti-chicken erythrocyte antibodies were used in the ADCC assay, but not for the AINC assay. The activity of the myeloperoxidase-hydrogen peroxide-halide antibacterial system of the neutrophil was evaluated by the iodination reaction with a 20-min incubation period, using opsonized zymosan as the stimulating particle, and ten percent

trichloroacetic acid to precipitate the bound iodine. Cytochrome C reduction was determined by means of a microtiter procedure with opsonized zymosan as the stimulant in a 5-min incubation period. The results were expressed as the optical density (550nm-650nm) per 2.5 x 10^6 PMNs. Random migration was evaluated by measuring the area of random migration under agarose after an 18-hr incubation period.

Statistical analysis

A mean and standard error of the mean were calculated for each neutrophil function value for each treatment group. An analysis of variance was used to determine the level of significance of the differences between control and genistein-treated cells.

RESULTS

A 3 hour pre-incubation of bovine neutrophils with genistein *in vitro*, caused a dose dependent decrease in superoxide anion (O_2^-) production (as measured by the cytochrome c reduction assay) and in the activity of the myeloperoxidase-hydrogen peroxide-halide system (as measured by iodination) when opsonized zymosan was used as the stimulus (Fig. 1,2). A concentration of 30 ug/ml of genistein, produced significant inhibition ($P < 0.05$) in both assays.

The mobility of genistein treated bovine neutrophils was investigated using a random migration under agarose assay. The results showed that bovine neutrophil random migration was inhibited by genistein treatment in a concentration dependent manner (Fig. 3). The lowest concentration tested that significantly inhibited random migration was 15 ug/ml ($P < 0.05$).

Genistein treatment (60 ug/ml) of bovine neutrophils significantly inhibited ($P < 0.05$) the percentage of ^{51}Cr released from the labeled cRBCs in the presence (ADCC) and absence of antibody (AINC) as compared to control cells (Fig. 4,5). Inhibition of ADCC and AINC required a higher concentration of genistein (greater than 30 ug/ml) than inhibition of cytochrome c reduction, iodination, or random migration.

As demonstrated above, O_2^- production by genistein (60

ug/ml) treatment of bovine neutrophils significantly decreased when using OZ as a stimulus. Interestingly, it was observed that O_2^- production by genistein (60 ug/ml) treatment of bovine neutrophils was not diminished but was significantly enhanced when using phorbol myristate acetate (PMA) as a stimulus (Fig. 6).

Fig.1 The effect of genistein on cytochrome c reduction by bovine neutrophils stimulated by bovine serum-opsonized zymosan. Neutrophils were incubated with the indicated concentration of genistein for 3 hours before stimulation. Data are expressed as mean ± SEM (n=19). $^{*}p < 0.05$.

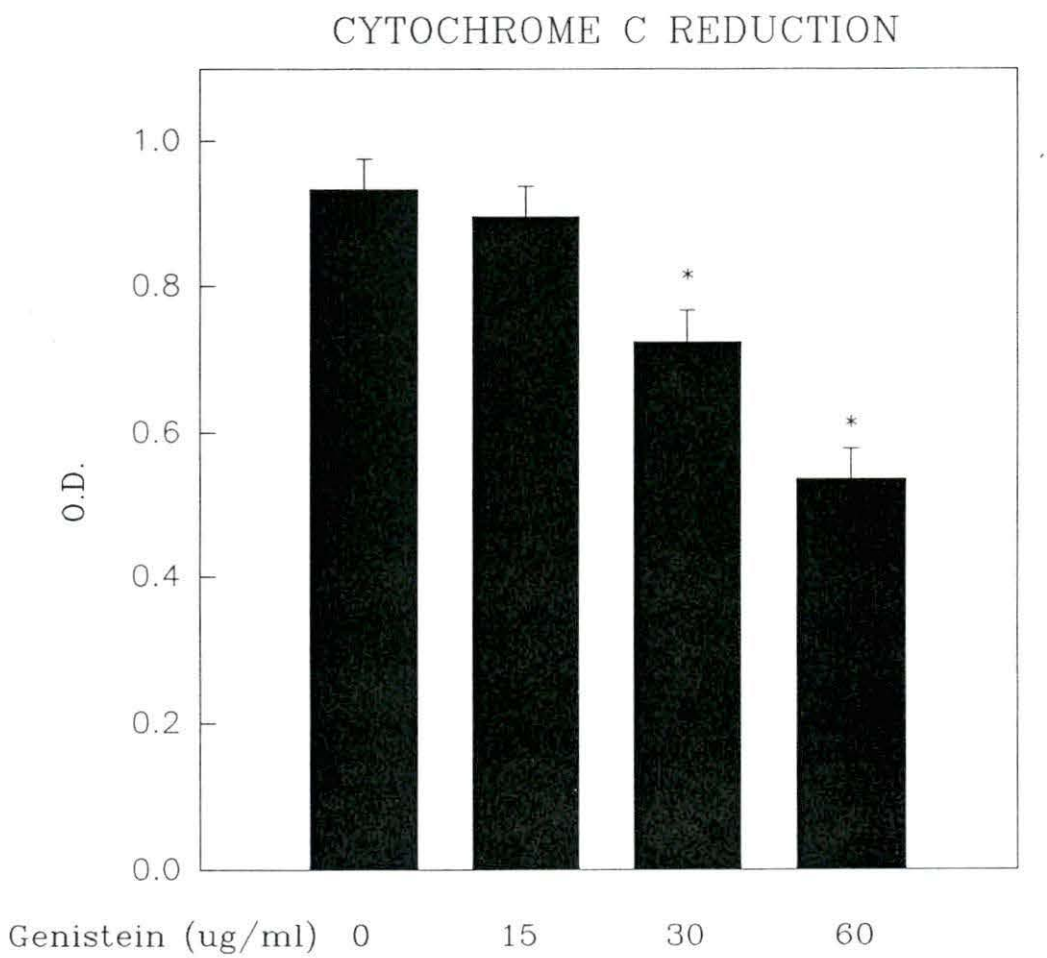

CYTOCHROME C REDUCTION
O.D.
1.0
0.8
0.6
0.4
0.2
0.0
*
*
Genistein (ug/ml) 0 15 30 60

Fig.2 The effect of genistein on iodination by bovine neutrophils stimulated by bovine serum-opsonized zymosan. Neutrophils were incubated with the indicated concentration of genistein for 3 hours before stimulation. Data are expressed as mean ± SEM (n=19). $^{*}p < 0.05$.

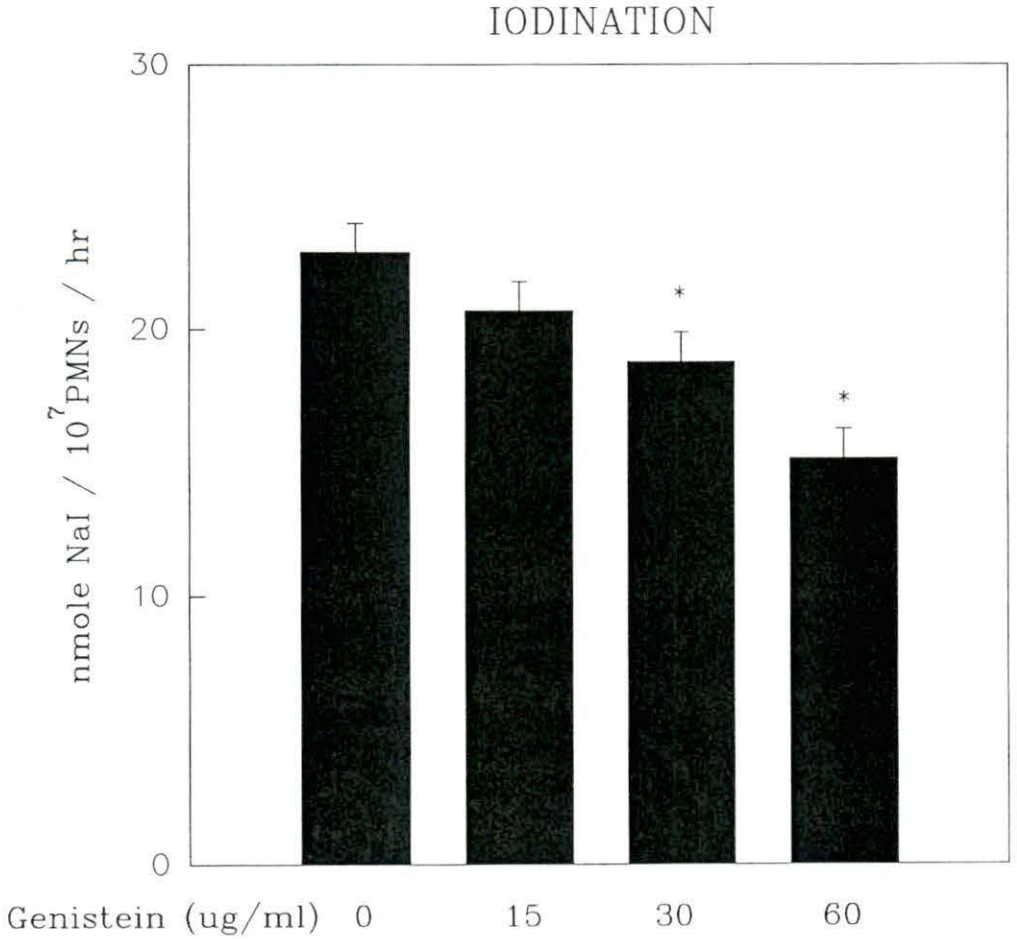

IODINATION
30
20
10
0
nmole NaI / 10^7 PMNs / hr
*
*
Genistein (ug/ml) 0 15 30 60

Fig.3 The effect of genistein on random migration by bovine neutrophils. Neutrophils were incubated with the indicated concentration of genistein for 3 hours before conducting the assay. Data are expressed as mean ± SEM (n=24). $^{*}p < 0.05$.

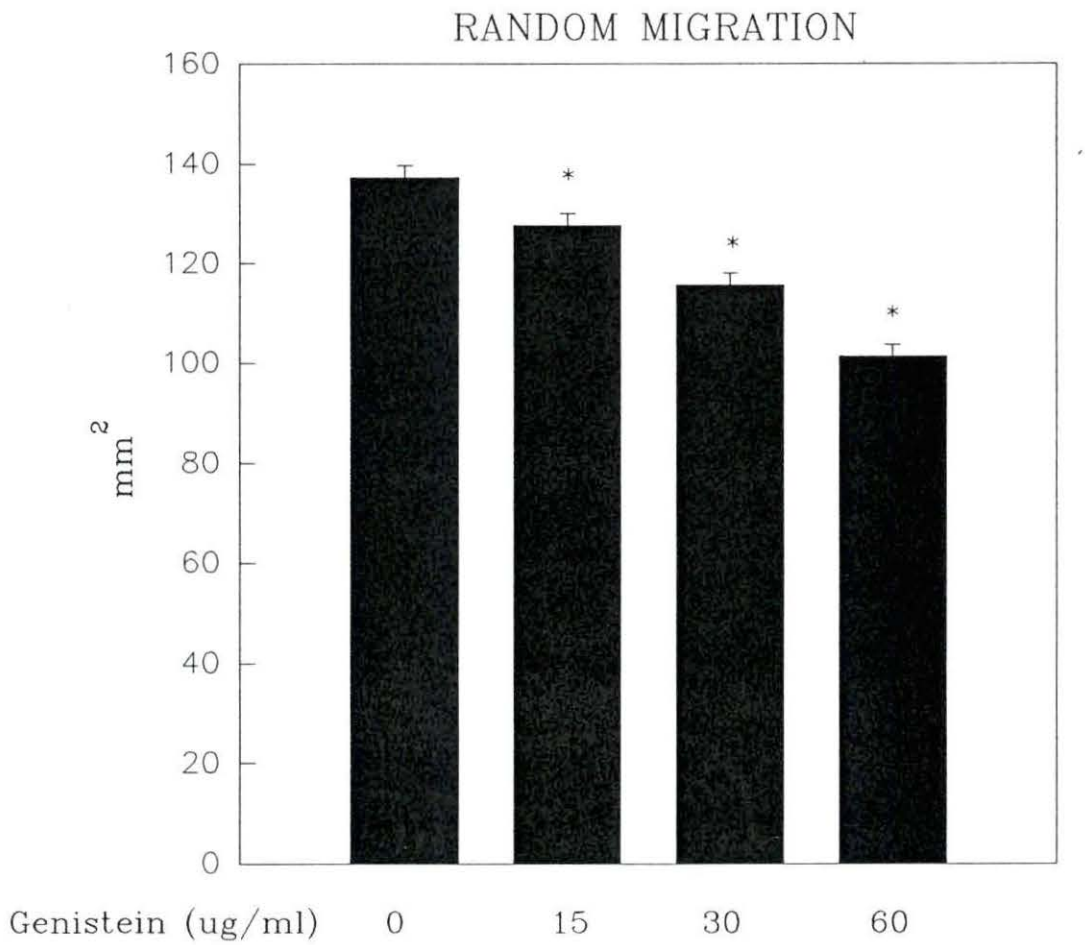

RANDOM MIGRATION
160
140
120
100
80
60
40
20
0
mm 2
*
*
*
Genistein (ug/ml)
0
15
30
60

Fig.4 The effect of genistein on antibody dependent cell-mediated cytotoxicity by bovine neutrophils. Neutrophils were incubated with the indicated concentration of genistein for 3 hours before conducting the assay. Data are expressed as mean ± SEM (n=24). $^{*}p < 0.05$.

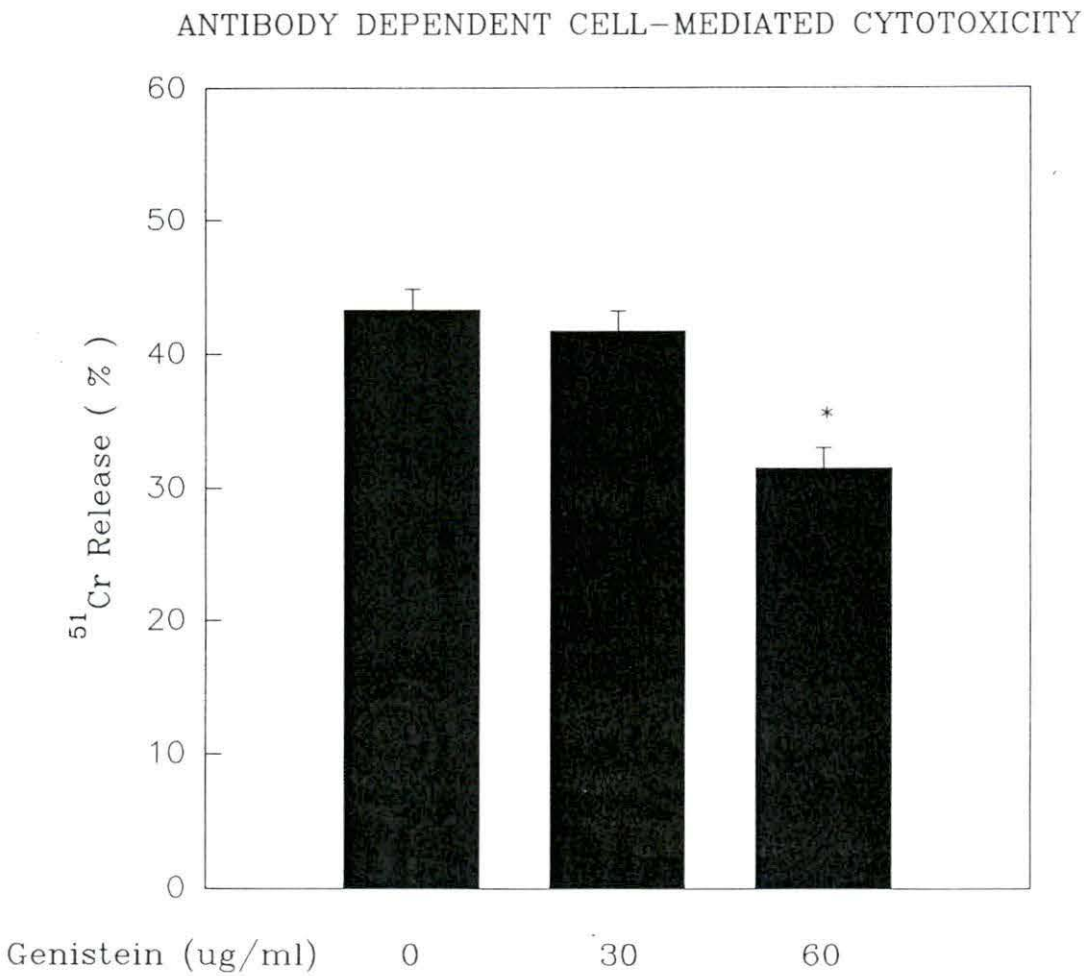

ANTIBODY DEPENDENT CELL-MEDIATED CYTOTOXICITY
60
50
40
30
20
10
0
^{51}Cr Release (%)
*
Genistein (ug/ml)
0
30
60

Fig.5 The effect of genistein on antibody independent cell-mediated cytotoxicity by bovine neutrophils. Neutrophils were incubated with the indicated concentration of genistein for 3 hours before conducting the assay. Data are expressed as mean ± SEM (n=24). $^{*}p < 0.05$.

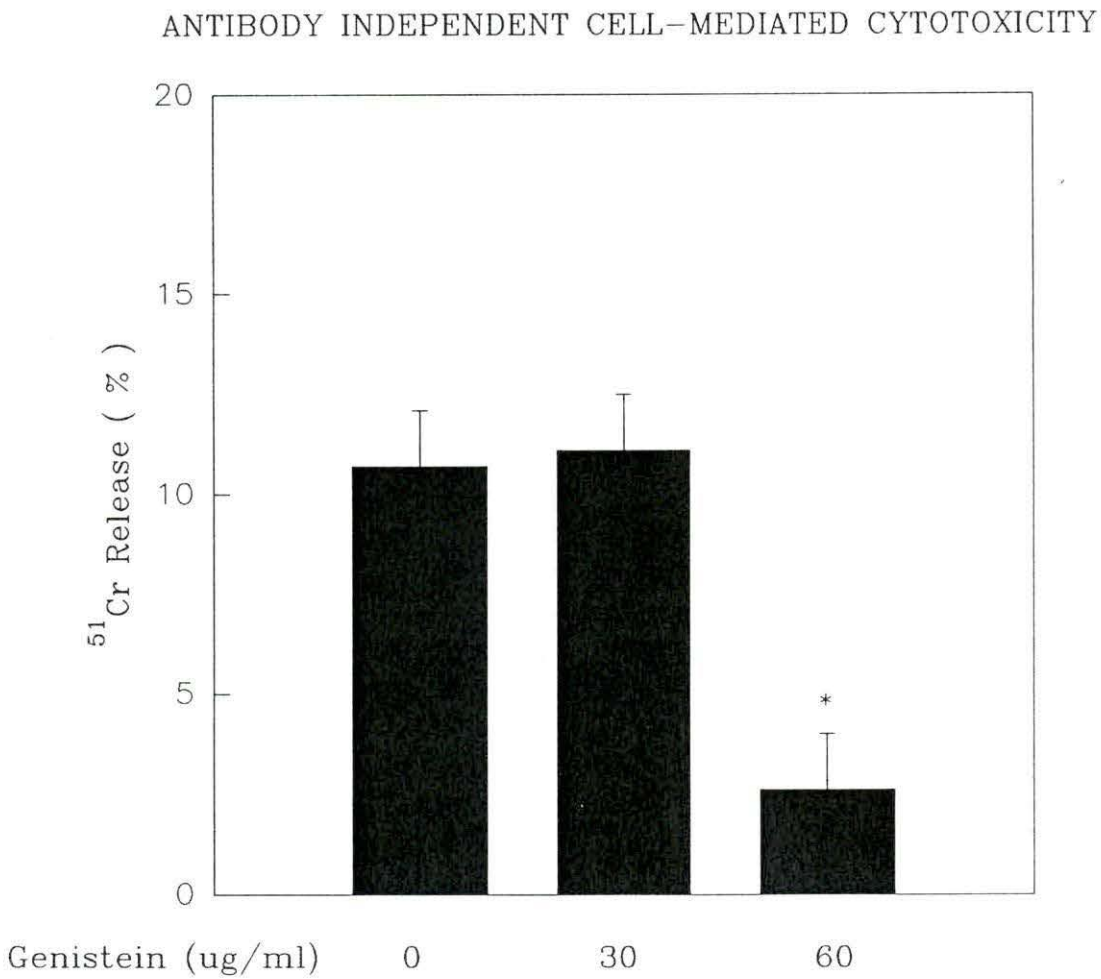
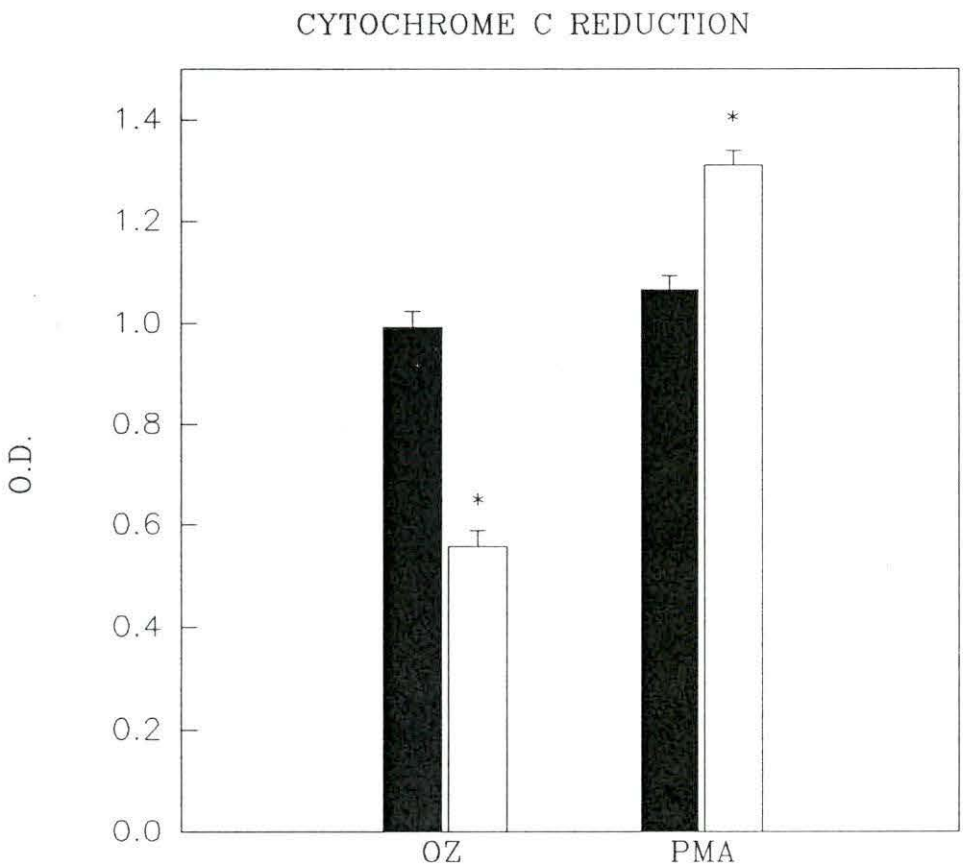

ANTIBODY INDEPENDENT CELL-MEDIATED CYTOTOXICITY
20
15
10
5
0
^{51}Cr Release (%)
*
Genistein (ug/ml)
0
30
60

Fig.6 Cytochrome c reduction by bovine neutrophils in response to bovine serum-opsonized zymosan and phorbol myristate acetate in the absence (solid bars) or presence (blank bars) of genistein pretreatment (60 ug/ml). Data are expressed as mean ± SEM (n=10). $^{*}p < 0.05$.


CYTOCHROME C REDUCTION
O.D.
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
*
OZ
PMA

DISCUSSION

In this study, superoxide anion (O_2^-) production (Fig. 1), and the activity of the myeloperoxidase-hydrogen peroxide-halide system (Fig. 2) in bovine neutrophils stimulated with opsonized zymosan (OZ) were diminished by <u>in vitro</u> genistein treatment. Spontaneous migration was also reduced by genistein treatment of bovine neutrophils in a dose-dependent manner (Fig. 3). Antibody dependent cell-mediated cytotoxicity and AINC were significantly inhibited by 60 ug/ml genistein (Fig. 4,5).

Superoxide anion production induced by OZ was inhibited by genistein (Fig. 1). In contrast, PMA-induced O_2^- production was not inhibited but was enhanced by genistein (60 ug/ml) pretreatment (Fig. 6). Previous studies have shown FMLP- and aggregated IgG-induced superoxide anion production by human neutrophils were inhibited by genistein; however, PMA- induced O_2^- production was neither inhibited nor significantly enhanced (Kusunoki et al. 1992). In the study by Kusunoki et al. (1992) human neutrophils were treated with 50 ug/ml of genistein for 30 minutes before the addition of various stimuli. However, different studies (Akiyama et al. 1987; Utsumi et al. 1992) reported that PMA-induced superoxide anion production was enhanced by low concentrations of genistein (9.5 ug/ml). The discrepancies in the results reported in these various manuscripts may be due to the different species

used as a source of neutrophils and different experimental systems.

Our observations suggest that: 1) The reduction of OZ-stimulated O_2^- production by genistein was not due to a generalized toxic effect of genistein on bovine neutrophils. There was no impaired cell viability as determined by trypan blue exclusion after various concentrations of genistein pretreatment. Furthermore, PMA-induced O_2^- production by bovine neutrophils was not inhibited by genistein pretreatment. 2) PKC activity and the steps leading to O_2^- production by NADPH oxidase after PKC stimulation do not involve protein tyrosine kinase activity. PKC was discovered as a receptor for PMA and is directly activated by PMA to induce O_2^- production by NADPH oxidase (Neidel et al. 1983; Wolfson et al. 1985). In the present study, O_2^- production was not inhibited by genistein pretreatment when using PMA as a stimulus, indicating that the signalling cascade following PKC activation leading to O_2^- production was not inhibited by genistein. 3) Protein tyrosine kinases are involved in OZ-induced O_2^- generation in a PKC-independent pathway or are involved in early stages of receptor-mediated signal transduction in a PKC-dependent pathway. Studies on human neutrophils by Maridonneau-Parini et al (1986) suggested that OZ-induced O_2^- generation was phospholipase A2 (PLA_2) mediated and independent of PKC activity. Recently, Watson et al. (1992) demonstrated that the initial (2 min - 3 min) phase of

O_2^- generation elicited by OZ was independent of PKC activity, whereas PKC was required for continuance of O_2^- generation in the latter phase (5 min - 30 min). In our observations, genistein inhibited OZ-induced O_2^- production but did not inhibit PMA-induced O_2^-production. Therefore, tyrosine kinase(s) may be involve in early steps before the activation of PKC or they may be involved in PKC independent pathway for O_2^- generation. The enhancement of PMA-induced O_2^- production by genistein- treated bovine neutrophils remains to be investigated. A possible explanation for this observation is that protein tyrosine kinases may play an inhibitory role in PKC-mediated NADPH oxidase activity.

In neutrophils, ADCC is initiated via the Fc receptor. A previous study has reported that FcγR crosslinking by aggregated IgG stimulated an increased phosphotyrosine content of intracellular proteins in human neutrophils (Connelly et al. 1991). The data presented here showed that genistein inhibited ADCC at a concentration of 60 ug/ml. The inhibition was not due to toxic effects of genistein, since there was no impaired cell viability as examined by trypan blue exclusion after treatment with genistein (60 ug/ml) for about 6h, and the genistein did not inhibit PMA induced O_2^- generation by bovine neutrophils as mentioned before in this study. Inhibition of ADCC by genistein (30 ug/ml, 4h) associated with inhibition of tyrosine phosphorylation was also observed in human natural killer (NK) cells (Stahls et al. 1992).

The ability of bovine neutrophils to mediate antibody independent cell-mediated cytotoxicity was reported by Lukacs et al. (1985) and is apparently similar to NK cell cytotoxicity (Trinchieri et al. 1989). The mechanism of this direct cell contact cytotoxicity is not yet defined. It was reported that NK-mediated cytotoxicity of K562 tumor target cells by either freshly isolated peripheral blood large granular leukocytes or cloned NK cells was inhibited by the tyrosine kinase inhibitors, herbimycin A and genistein (Einspahr et al. 1991). Our data demonstrated that AINC against cRBC by bovine neutrophils was significantly decreased at a concentration of 60 ug/ml genistein (Fig. 5). These results suggest the involvement of protein tyrosine kinases in FcR-mediated and direct cell contact-mediated cytotoxicity in bovine neutrophils.

Genistein-treated bovine neutrophils had inhibited random migration (Fig. 3). In human neutrophils, random migration was found to be impaired by another protein tyrosine kinase inhibitor, erbstatin (Gaudry et al. 1992). Human neutrophil adherence to fetal bovine serum(FBS)-coated surfaces in the presence of TNF caused tyrosine phosphorylation of several endogenous proteins. Genistein (8 ug/ml) blocked neutrophil spreading when neutrophils were plated on FBS-coated glass coverslips and were stimulated with TNF (Fuortes et al. 1993). In the random migration under agarose assay used in this study, neutrophils adhered to and migrated along a plastic

surface. Our observation that random migration was inhibited by genistein suggests that the non-specific adherence to plastic and non-directed migration by neutrophils involve the activity of a protein tyrosine kinase(s) for the reorganization of the cytoskeletal system by an undefined mechanism.

Recently, two studies (Huang et al. 1992; Young et al. 1993) reported that the inhibitory effect of genistein on a protein histidine kinase (ID_{50} = 27 ug/ml) and on protein kinase-independent events (lactate transport in isolated rat erythrocytes (ID_{50} = 2.3 ug/ml), respiratory chain activity in an isolated mitochondria system (ID_{50} = 18.9 ug/ml), and aldehyde dehydrogenase in a purified enzyme system (ID_{50} = 8.1 ug/ml). However, the involvement of protein tyrosine kinase(s) in the mechanism of lactate transport in isolated erythrocytes and of ATP production by an isolated mitochondria system can not be ruled out. There is apparently no published information about the roles of protein histidine kinase and aldehyde dehydrogenase in neutrophil functions. In addition, after a 3h preincubation of bovine neutrophils with various concentrations of genistein, the exact concentration of genistein getting into intact neutrophils is not known. Therefore, the potential role for genistein in inhibiting bovine neutrophil function through inhibition of protein histidine kinase and aldehyde dehydrogenase is not known.

In summary, the evidence presented in this study using

the protein tyrosine kinase inhibitor genistein suggests that tyrosine kinases are involved in bovine neutrophil function. Further studies need to be done to confirm and define the role of protein tyrosine kinases in neutrophil function.

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GENERAL SUMMARY

The data presented in the first paper of this thesis demonstrated that several aspects of neutrophil function, including oxidative metabolism, myeloperoxidase-hydrogen peroxide-halide activity, chemotaxis and random migration, were significantly decreased in young calves (2-4 months) as compared to mature cattle (2-3 years). There was no significant difference in cytoplasmic Ca^{2+} flux in response to bovine serum opsonized-zymosan (BOZ) stimulation between the two age groups. Neutrophils from young calves also had decreased myeloperoxidase activity and were smaller than neutrophils from adult cattle.

Recombinant bovine IFN-γ (rboIFN-γ) had similar *in vitro* influence on the neutrophils from both age groups. Enhancement of ADCC and oxidative metabolism with rboIFN-γ treatment were observed in the neutrophils from both young calves and adult cattle. RboIFN-γ treatment *in vitro* inhibited chemotaxis, migration and Ca^{2+} mobilization by neutrophils from both age groups. In contrast, it has been reported that recombinant human IFN-γ can correct the decreased Ca^{2+} mobilization observed in human neonates (Hill 1993).

Both BOZ-stimulated and phorbol myristate acetate (PMA)-stimulated calf neutrophils had decreased oxidative metabolism

and myeloperoxidase-hydrogen peroxide-halide activity. The decreased myeloperoxidase activity observed in neutrophils from young calves was probably at least partially responsible for the decreased myeloperoxidase-hydrogen peroxide-halide activity observed.

It was also of interest to know if there were other defective factors resulting in impaired signal transduction in neutrophils from young animals as compared to adult animals. Therefore the objective of the second paper in this thesis was to demonstrate the role of protein tyrosine kinase(s) in neutrophil function using genistein as a tyrosine kinase inhibitor. Data presented in this experiment showed genistein inhibited ADCC, AINC, myeloperoxidase-hydrogen peroxide-halide activity, and random migration. Oxidative metabolism in response to BOZ was inhibited by treatment of bovine neutrophils with genistein , but was not inhibited in response to PMA. This suggests that the involvement of protein tyrosine kinase(s) in several aspects of bovine neutrophil function.

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The poem cited below is my favorite child poem:

Magical People-The Little Elf

I met a little elf man once. Down where the lilies blow.
I asked him why he was so small and why he didn't grow.
He slightly frowned, and with his eyes,
He looked me through and through
" I'm quite as big for me" said he
" As you are big for you."
-John Kendrick Bangs-

I wish to thank the Lord. Through the grace of his love, I have learned to be myself and to persist in what I stand for. I am grateful to my parents for their unconditional love and support. I wish to thank Dr. Roth, my major professor, for his opinions and patience in instructing my writing when I was working with this thesis. I especially express my thanks to Dr. Dagmar Frank for her help with my research. I also thank my committee (Dr. Kaeberle, Dr. Graves, Dr. Myers, and Dr. Nilsen-Hamilton) for their time. At last, I wish to thank my dear friend William J. Gale for his care and friendship.