Regulation of degranulation of bovine neutrophils

by

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GENERAL INTRODUCTION

Introduction

Neutrophils are a type of white blood cell that is critical in the defense of the host against bacterial infection. Neutrophils contain membrane bound vesicles called granules or lysosomes. The granules contain bactericidal substances, degradative enzymes, and many other components. Several granule types, with varying contents and functions, have been described. There are differences among species in the types of granules contained within the neutrophils. The granules fuse with and release their antimicrobial contents into phagosomes that contain engulfed microbes or to the exterior of the cell. This process is called degranulation. A number of agents are able to inhibit the degranulation process, which may lead to an increased risk of bacterial infection. Granule contents are vital to the destruction of bacteria, but may also cause damage to host tissues. Neutrophils have been implicated in causing injury to normal tissues by excessive discharge of their granules, or degranulation at inappropriate times. If regulation of neutrophil degranulation were sufficiently understood, it might be possible to design drugs to enhance granule release to combat infection or inhibit degranulation to prevent unnecessary tissue damage.

Regulation of bovine neutrophil large granules

A distinguishing structural characteristic of ruminant neutrophils, as compared to other species, is the presence of a unique third granule type (Gennaro et al., 1983). Ruminant neutrophils have peroxidase positive granules that form early in granulopoiesis, and peroxidase negative granules of similar size that form late in cell development. These granule types have the characteristics of primary or azurophilic and secondary or specific granules seen in other species. The unique granule population of ruminant neutrophils, called the large granules, is formed after the primary granules but before the secondary granules during cell differentiation. These granules contain unique potent oxygen-independent cationic antimicrobial peptides, which are called bactenecins. Large granules also contain lactoferrin, but do not contain peroxidase, lysosomal hydrolases, or other substances found in the other granule types (Baggiolini et al., 1985).

Bactenecins are believed to be an important component of ruminant defense against bacterial infections. Three distinct bactenecin molecules have been reported. Two of these peptides, called Bac5 and Bac7, are able to kill several species of bacteria that are potentially pathogenic for cattle, including *E. coli*, *Salmonella typhimurium*, *Klebsiella pneumoniae* and *Leptospira interogans*. They are not bactericidal for *Staphylococcus aureus*, *Streptococcus agalactiae*, or *Borrelia burgdorferi*. The third bactenecin, a cyclic dodecapeptide, is bactericidal for *S. aureus* and *E. coli*. Bactenecins have antiviral properties as well; they have been shown to inactivate

human herpes simplex virus types 1 and 2 (Romeo et al., 1988; Gennaro et al., 1989; Scocchi et al., 1993).

The objective of this research was to characterize factors that regulate large granule discharge. We hypothesized that immunosuppressive agents and bacterial or viral virulence factors may inhibit large granule degranulation, thereby increasing the animal's susceptibility to bacterial infection. We further hypothesized that selected cytokines and growth factors may enhance degranulation in normal neutrophils, and may enable suppressed neutrophils to overcome inhibition of degranulation.

The first specific goal of this research was to develop an assay to measure the release of bactenecins as a marker for large granule degranulation. Using the published amino acid sequence of Bac7 (Frank et al., 1990), the amino terminal 20 amino acid portion was predicted to be the most strongly antigenic part of the peptide. The 20 amino acid portion of the molecule was synthesized by the protein facility at Iowa State University. Peptides of such low molecular weight are generally non-immunogenic, so the synthetic peptide was conjugated to hemocyanin of keyhole limpets. Once conjugated to the much larger protein, the synthetic peptide should have become antigenic as a hapten. The conjugated protein and an adjuvant were injected into rabbits to produce specific anti-bactenecin polyclonal antibody. Several methods of antibody purification were used in an attempt to remove cross-reactive antibodies, leaving only antibodies specific for bactenecins or their precursor molecules.

To determine the amount of Bac7 released from stimulated neutrophils, an adaptation of a previously described quantitative dot-blot was attempted (Glenney et al., 1982; Zanetti et al., 1991). Briefly, measured quantities of cell-free supernatants from neutrophil preparations were blotted onto nitrocellulose squares. The squares were probed for the presence of bactenecins using polyclonal antibody. Unbound antibody was removed by washing, and the squares were incubated with ^{125}I-labeled protein A. Unbound protein A was washed away, and the radioactivity of each square was measured in a gamma counter. The amount of bactenecin discharged was reported as a percent of total neutrophil bactenecin content determined using lysed neutrophils.

The second specific goal of this research was to determine which stimulants of neutrophil activity result in release of bactenecins or probactenecins. The peptides are stored in large granules as inactive probactenecins. Probactenecins must be cleaved by elastase from the primary granule to form the mature active form of the peptide (Scocchi et al., 1992). Stimulants that cause degranulation of only large granules cause release of probactenecins. Stimulants that induce degranulation of both large and primary granules allow the mixing of granule contents, which enables elastase to cleave the probactenecins, resulting in discharge of the mature active bactenecins. It is reported that phorbol myristate acetate induces degranulation of large granules, while opsonized bacteria cause degranulation of both large and primary granules (Zanetti et al., 1991). Other known stimulants of neutrophil activity, for example platelet activating factor and calcium ionophore, were to be

tested to determine their effect on large and primary granules. Data of this type would aid in understanding the intracellular signaling pathways involved in the regulation of selective degranulation.

A third goal was to determine if glucocorticoids or other inhibitors of neutrophil degranulation, such as bacterial factors, suppress degranulation of large granules. Steroids and bacterial products have been shown to suppress many other functions of neutrophils, including primary granule degranulation. Neutrophils isolated from healthy adult cattle treated with immunosuppressive doses of dexamethasone would be stimulated in vitro to cause large granule discharge. The amount of release of bactenecin would be compared with neutrophils from untreated animals to determine what effect, if any, dexamethasone has on large granule degranulation. Bacteria can produce factors that suppress degranulation. For example, *Haemophilus somnus* and *Brucella abortus* release adenine and guanine nucleotides that have been shown to inhibit primary granule degranulation (Canning et al., 1986; Chiang et al., 1986; Bertram et al., 1986). Isolated neutrophils would be exposed in vitro to *Haemophilus somnus* supernatants or adenine and GMP, and bactenecin discharge compared to untreated neutrophils from the same animal. This information would indicate if such agents might increase susceptibility to bacterial infection by reducing degranulation of large granules.

A fourth goal of this research was to determine if cytokine or growth factor treatment of neutrophils enhances bactenecin release or normalizes inhibition of degranulation. Several cytokines are important enhancers of neutrophil functions,

including interleukin-1, interleukin-8, interferon gamma, and granulocyte-macrophage colony-stimulating factor. Isolated neutrophils from untreated and dexamethasone-treated cattle would be exposed to recombinant human cytokines (recombinant bovine cytokines when available) in vitro to determine what factors directly induce degranulation, enhance neutrophil response to stimulants, or reverse the inhibitory effects of dexamethasone. Isolated neutrophils would be exposed to cytokines concurrently with *H. somnus* factors or adenine and GMP to see if cytokines can reverse the inhibition of degranulation due to bacterial products.

Regulation of bovine neutrophil primary granules

A separate objective of this research was to develop an assay to directly and quantitatively measure degranulation of bovine neutrophil primary granules. Several assays have been described to measure release of primary granule contents, such as elastase and beta-glucuronidase. These assays are labor and equipment intensive, require extended incubation periods, or use toxic or radioactive substances. Our group has used the iodination assay as a measure of primary granule discharge. This assay uses radioactive material, and is an indirect measure of primary granule release. Iodination measures the ability of the myeloperoxidase-H_2O_2-halide antibacterial system of neutrophils to incorporate ^{125}I into protein. Iodination may be reduced by

an inhibition of degranulation, but several other factors may also affect the ability of neutrophils to iodinate protein (Roth and Kaeberle, 1981).

An assay to measure myeloperoxidase release from human neutrophil primary granules has recently been described (Menegazzi et al., 1992). It is rapid, direct, quantitative, and uses no radioactive or highly toxic substances. We were able to adapt this assay for use with cattle neutrophils. The originally described assay was altered in several ways, in part because cattle neutrophils contain significantly less myeloperoxidase than human neutrophils. The assay employs a sensitive and relatively non-toxic peroxidase substrate to measure extracellular myeloperoxidase. When primary granules discharge to the cell exterior, myeloperoxidase in conjunction with H_2O_2 can oxidize the substrate, causing a color change. Supernatants can then be separated from the stimulated neutrophils, and the optical density of the color change is quantified using an ELISA reader.

Brucella abortus and *Haemophilus somnus* inhibit neutrophil iodination by releasing adenine and GMP (Canning et al., 1986; Chiang et al., 1986). These nucleotides inhibit degranulation of primary granules (Bertram et al., 1986). In order to validate the myeloperoxidase assay, isolated neutrophils were stimulated to degranulate in vitro using opsonized particles as the stimulant. Increasing concentrations of adenine and GMP were added to the neutrophil preparations. The nucleotides suppressed myeloperoxidase discharge in a linear manner similar to the effect previously reported. The results of the myeloperoxidase assay supported the proposed mechanism of *H. somnus* and *B. abortus* inhibition of iodination.

Neutrophils from calves have a number of functional deficits compared to neutrophils from adult animals, including reduced iodination. It has been postulated that this reduced iodination may be due to decreased myeloperoxidase content of calf neutrophils. However, there are conflicting reports regarding total myeloperoxidase content of calf neutrophils compared to adult neutrophils (Hauser et al., 1986; Lee and Roth, 1992; Zwahlen et al., 1992). The total myeloperoxidase content of a given number of lysed neutrophils from calves and adults was quantitated using the myeloperoxidase assay. The kinetics of primary granule degranulation for each age group were also examined, apparently for the first time. Using the newly adapted assay of myeloperoxidase, it was determined that adult cattle neutrophils contain significantly more total myeloperoxidase than calf neutrophils. However, neutrophils from calves were more responsive to stimulation by opsonized particles, degranulating more rapidly and more completely than neutrophils from adult animals.

Thesis organization

This thesis describes the regulation of degranulation of neutrophils, and our attempts to augment that knowledge. Following this general introduction is a literature review, two manuscripts prepared for submission to journals, and a general conclusions chapter. The literature review consists of a review of published literature regarding primarily human and cattle neutrophils and their granules. Subjects

included in the review are a description of neutrophil granules and their contents, methods to measure the discharge of the different granule types, physiology and signal transduction of degranulation, and a discussion of agents that stimulate, suppress, or enhance degranulation. The first manuscript describes the myeloperoxidase assay that was adapted to measure primary granule release in cattle. Included are data that were obtained regarding various stimulants of primary granule exocytosis, the effect of adenine and GMP on degranulation, the total myeloperoxidase content of neutrophils from calves and adults, and the kinetics of degranulation of the primary granule for calves and adults. This manuscript has been submitted for publication. The second manuscript describes our attempts to measure secretion of bovine large granules. This manuscript reports on the creation of the synthetic bactenecin, production of polyclonal antibodies to the synthetic peptide, and attempts to purify antibodies and develop an assay to measure cattle neutrophil release of the third granule.

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LITERATURE REVIEW

Contents of neutrophil granules

Neutrophils are vital in protection of the host against bacterial infection. They provide a first line of rapidly responding non-specific defense. Neutrophils possess a number of antibacterial mechanisms that may be divided into two categories. The first system, often called the respiratory burst, kills microbes by production of toxic oxygen compounds, such as superoxide radical, hydroxyl radical, and hydrogen peroxide. The second category of antibacterial components consists of preformed, oxygen independent cytotoxic compounds. These substances are enclosed within membrane bound vesicles within neutrophils referred to as granules or lysosomes (Gennaro et al., 1991). When a neutrophil ingests an object into a phagosome, these granules can fuse with the phagosome and discharge their contents into it. Alternatively, granules may fuse with the cell membrane and expel their contents to the exterior of the cell (Henson et al., 1992). In either location, liberated peptides, enzymes, and hydrolases can attack the microbes. As the neutrophil-microbe interaction progresses, the pH inside the phagolysosome or outside the cell may be reduced due to microbial and host factors. Neutrophil granules contain various enzymes and hydrolases that are optimally active at different pH levels to ensure bactericidal activity over a broad pH range. In addition to antimicrobial substances,

some granule types contain readily mobilized adhesion molecules and enzymes that aid the neutrophils in leaving the circulation and migrating through tissues and matrix substances (Sengelov et al., 1993).

Neutrophils from various species may have different identifiable types of granules that may contain distinct mixtures of contents. Three types of granules have been described in bovine neutrophils, and at least four in human neutrophils. Primary (or azurophilic) and secondary (or specific) granules are common to both species. Bovine neutrophils contain a unique granule which has been called the large granule (Gennaro et al., 1983a). Human neutrophils contain two small granule types called gelatinase granules and secretory vesicles (Sengelov et al., 1993).

The first type of granule to form during development of neutrophils is the primary or azurophilic granule (Baggiolini et al., 1985; Bainton, 1992). Cattle primary granules are strongly peroxidase-positive, usually rod-shaped, and relatively small and fewer in number compared to primary granules in human neutrophils (Baggiolini et al., 1985). Bovine primary granules contain myeloperoxidase, elastase, catalase, beta-glucuronidase, and beta-galactosidase, all at lower levels than human neutrophils, as well as a number of degradative enzymes such as acid and neutral hydrolases and nucleases (Gennaro et al., 1978). Cattle neutrophil primary granules contain little or no lysozyme (Gennaro et al., 1978; Bertram, 1985). Human neutrophil primary granules have these same enzymes as well as cathepsins, relatively large amounts of lysozyme, bactericidal/permeability-increasing protein, cationic

antimicrobial peptides known as defensins, plus many other substances (Gennaro et al., 1991; Borregaard et al., 1995).

Secondary or specific granules form late in cellular differentiation (Baggiolini et al., 1985; Bainton, 1992). They are peroxidase negative and about the same size as primary granules. In cattle neutrophils, they contain lactoferrin, type I collagenase, vitamin B_{12}-binding protein (Gennaro et al., 1991), and preformed membrane-bound Mac-1 (CD11b/CD18) adhesion molecules (Ackermann et al., 1993; Burton and Kehrli, 1995a). Human neutrophil secondary granules contain these same substances, but also contain receptors for the synthetic peptide FMLP (N-formyl-methionyl-leucyl-phenylalanine), fibronectin and vitronectin receptors, TNF receptors, and other substances (Bainton, 1992; Borregaard et al., 1995).

Ruminant neutrophils possess a unique granule type known as the large granule. It is morphologically larger and denser than the other granule types and is peroxidase negative. It is the predominant granule type in cattle neutrophils (Gennaro et al., 1983a). During cell development, large granules are formed after primary granules but before secondary granules (Baggiolini et al., 1985). Large granules contain lactoferrin (Gennaro et al., 1991) and potent antimicrobial peptides known as bactenecins (Romeo et al., 1988; Gennaro et al., 1989). Bactenecins (three different ones have been described) are stored within the granules as inactive pro-peptides. When large granules fuse with phagolysosomes or secrete to the cell exterior, elastase released from primary granules proteolytically cleaves the pro-bactenecins to their active forms (Zanetti et al., 1990; Zanetti et al., 1991; Scocchi et

al., 1992). Two of the bactenecins, Bac5 and Bac7, have been well described. Bac5 is a 42 amino acid peptide, and Bac7 is 59 amino acids long. Both proteins consist of greater than 45% proline and greater than 23% arginine, but their sequences are distinct from each other (Frank et al., 1990). They have been shown to be able to kill several types of Gram-negative bacteria and spirochetes, and can inactivate human herpes simplex virus types 1 and 2 (Gennaro et al., 1983b; Romeo et al., 1988; Gennaro et al., 1989; Scocchi et al., 1993). The third bactenecin is a cyclic peptide of twelve amino acids, consisting predominantly of arginine and valine. It is bactericidal for *E. coli* and *Staphylococcus aureus* (Romeo et al., 1988).

Granule fractions from bovine neutrophil lysates contain other bactericidal peptides called indolicidin (Selsted et al., 1992) and beta-defensins (Selsted et al., 1993). Apparently, the subcellular location of these peptides has not been identified. Indolicidin is a 13 amino acid peptide that is unique to bovine neutrophils. Five of the amino acids are tryptophan, three are proline, and two arginine. Indolicidin has the highest mole percent of tryptophan among known protein sequences (Selsted et al., 1992). The beta-defensins are a group of cationic 38-42 amino acid peptides that has been recently described (Selsted et al., 1993). Each of the peptides contains six cysteine residues whose intrachain location is constant among the peptides. These cysteines form three disulfide bonds, giving each of the peptides a similar conformation. Beta-defensins are similar in structure and function to defensins from human neutrophils, but are distinct in their amino acid sequences and disulfide bond motifs (Tang and Selsted, 1993).

Two other discrete granule types have been described in human neutrophils. Gelatinase granules contain gelatinase, FMLP receptors, Mac-1, and other functional molecules (Borregaard et al., 1995). Secretory vesicles hold alkaline phosphatase, complement receptor I, Mac-1, FMLP receptor, Fc-gamma receptor III, and other substances (Borregaard et al., 1995). While these compartments seem not to contain substances distinct from the other granule types, they can be distinguished from the other granule types by their differing regulation of secretion (Sengelov et al., 1993; Sengelov et al., 1995). The existence of another distinct compartment in human neutrophils containing complement receptors has been proposed (Brown et al., 1991).

Several genetic defects of neutrophil granules and granule contents have been described in humans and in cattle. These defects help illustrate the importance of normal neutrophil granule functions; most contribute to an increased risk of recurrent infection in affected individuals, with potentially lethal results for some defects. Human neutrophil granule defects that have been noted include reduction or lack of primary granules, reduction or lack of secondary granules, and incomplete granule contents, such as deficiency of myeloperoxidase, alkaline phosphatase, lactoferrin, or defensins. A condition similar to human Chediak-Higashi syndrome has been observed in partially albino Hereford cattle (Bainton, 1992; Gallin, 1992; Shuster et al., 1992). Chediak-Higashi syndrome includes defects related to disorders of microtubule assembly. These defects affect several cell types. In neutrophils, the defects result in formation of giant granules within the neutrophils, principally from fusion of primary granules, but secondary granules may also be involved. The defects

cause impaired neutrophil migration and defective degranulation, which result in an increased risk of bacterial infection (Gallin, 1992). Myeloperoxidase deficiency is an exception to the generality that neutrophil dysfunction causes recurrent infections. In myeloperoxidase deficiency, minimal effects are noted unless some other neutrophil defect is also present (Bainton, 1992).

Assays of neutrophil degranulation

While genetic defects of neutrophils and granules can yield insights as to the function and importance of neutrophil granules, normal cells must be studied to understand how degranulation is regulated. To study the degranulation and differential regulation of the different granule types, one needs to measure release of a substance that is unique to the granule type of interest, examine fixed neutrophils microscopically (Bertram et al., 1986), or devise some other method to identify discharge of a specific compartment (Sengelov et al., 1995). Because of these conditions, relatively few of the many potential markers of degranulation have been assayed (Smolen, 1989). For studies of primary granule degranulation, myeloperoxidase (Suzuki et al., 1983; Menegazzi et al., 1992; Sengelov et al., 1995), beta-glucuronidase (Rausch and Moore, 1975; Buchta, 1990; Watson et al., 1995b), elastase (Sklar et al., 1982; Brown and Roth, 1991), lysozyme (human neutrophils) (Ferrante et al., 1988), and iodination (Roth and Kaeberle, 1981b) have been used.

Secondary granule markers that have been assayed include vitamin B_{12}-binding protein (Gennaro et al., 1978), lactoferrin (Gennaro et al., 1978), and CD18 (Burton et al., 1995). The bactenecins Bac7 and Bac5 have been used as markers to detect secretion of bovine large granules (Zanetti et al., 1990; Zanetti et al., 1991). Exocytosis of gelatinase granules has been studied by measuring gelatinase release (Sengelov et al., 1993), and secretory vesicle secretion has been measured by assaying alkaline phosphatase release (Borregaard et al., 1995). Flow cytometry also has been used to indirectly determine degranulation (Smolen, 1989).

In cattle, primary granule degranulation has been measured using microscopic morphometry (Bertram et al., 1986), the iodination assay (Roth and Kaeberle, 1981b), and release of beta-glucuronidase (Watson et al., 1995b), elastase (Brown and Roth, 1991), and myeloperoxidase (MPO) (Zwahlen et al., 1992; Quade and Roth, 1996). Each of these assays has advantages and disadvantages. Morphometry is labor and equipment intensive; measuring multiple samples would be very difficult. The iodination assay is an indirect measure of primary granule degranulation. The assay determines the ability of the myeloperoxidase-H_2O_2-halide system to incorporate radio-labeled iodine into tyrosine residues of proteins. This involves a complex series of events, and could be affected by disorders of normal oxidative mechanisms, reduced degranulation, reduced MPO content of the primary granule, lack of production or destruction of H_2O_2, or interference with the chemical reaction catalyzed by MPO (Roth and Kaeberle, 1981c). Measurements of beta-glucuronidase require extended incubation periods (Watson et al., 1995b). The elastase assay is

labor and equipment intensive, and does not lend itself to measuring multiple samples (Brown and Roth, 1991).

Several myeloperoxidase assays have been described. Myeloperoxidase is a highly cationic protein that tends to stick to cell membranes and test tube walls. Assays that measure MPO in supernatants of degranulating neutrophils tend to underestimate the actual MPO release (Menegazzi et al., 1992). Some MPO assays use toxic substrates (Suzuki et al., 1983; Menegazzi et al., 1992). Assays have been described that address the problems of toxic reagents, long incubation times, expensive labor and equipment, and MPO "stickiness" (Menegazzi et al., 1992; Quade and Roth, 1996). Briefly, a relatively non-toxic peroxidase substrate, tetramethyl benzidine, is added to microtiter plate wells containing neutrophils that have been incubated with stimulants. By adding substrate before separating supernatants from cells, MPO in supernatants plus MPO adhered to cells and plate wells should actively cleave the substrate and be measured. After a short incubation, sulfuric acid is added to stop the reaction and create a color change. Supernatants are separated from the cells by centrifugation, and the optical density of supernatant from each well is quantitated using an ELISA reader. This method is rapid, quantitative, and is a direct measure of primary granule exocytosis (Menegazzi et al., 1992; Quade and Roth, 1996). An ELISA assay using monoclonal antibodies to human MPO to measure human neutrophil myeloperoxidase release has been recently described (Sengelov et al., 1993; Sengelov et al., 1995). Monoclonal antibodies against bovine myeloperoxidase have been developed (Cooray et al., 1995),

but apparently no ELISA for bovine neutrophil myeloperoxidase has been described. ELISA assays may underestimate MPO exocytosis due to the stickiness of the enzyme.

Degranulation of the secondary or specific granule of human and bovine neutrophils has been measured by assaying for the exocytosis of vitamin B_{12}-binding-protein (Smolen, 1989; Buchta, 1990; Watson et al., 1995b). Lactoferrin and lysozyme have been used to determine human neutrophil specific granule release (Smolen, 1989). However, these assays are of limited value in cattle, because bovine neutrophils lack lysozyme (Gennaro et al., 1978), and lactoferrin is found in large granules as well as in specific granules (Gennaro et al., 1991).

Degranulation of bovine neutrophil large granules has been determined using a quantitative dot-blot technique (Zanetti et al., 1991). Polyclonal antibodies were produced that recognized either Bac5 or Bac7. Supernatants from degranulating neutrophils were blotted onto nitrocellulose squares, which were then exposed to polyclonal antibody. Unbound antibody was washed away, and the blots were then exposed to ^{125}I-labeled protein A. Unbound protein A was washed off, and the radioactivity of bound protein A was determined using a gamma counter.

Gelatinase granules and secretory vesicles are found in human neutrophils but have not been identified in bovine neutrophils. Assays to determine degranulation of these compartments have been described (Sengelov et al., 1993; Sengelov et al., 1995). Alkaline phosphatase was used as a marker of secretory vesicle release. Alkaline phosphatase is also found in neutrophil plasma membranes; membrane alkaline phosphatase was measured in the absence of detergent, while secretory

vesicle enzyme was determined in the presence of detergent. Gelatinase secretion by gelatinase granules was measured by ELISA.

The physiology of degranulation

In order to discharge their contents, granules must be able to move through the cytoplasm, fuse with the phagosomal or plasma membrane, and evaginate. The process of vesicle movement and fusion is poorly understood. Research examining vesicle movement and degranulation frequently uses permeabilized human neutrophils (Niessen et al., 1994), neutrophil-like cell lines (Cockcroft et al., 1994), or other cell types that exhibit functions analogous to neutrophil degranulation, for example mast cells and adrenal chromaffin cells (Gomperts, 1990). Some of the information obtained using these different cell types may not apply to normal physiological degranulation in bovine neutrophils.

Movement of granules requires a functional cytoskeleton, and microtubule and microfilament polymerization (Bertram, 1985). F-actin and fodrin appear to be especially important (Henson et al., 1992). Intracellular calcium levels, and calcium-dependent proteins such as the annexin family (Creutz, 1992; Blackwood and Hessler, 1995), calmodulin, and protein kinase C (Henson et al., 1992) play important roles in granule movement, membrane attachment, and membrane fusion. These proteins are found consistently in cell types capable of exocytosis and show considerable sequence

homology among the various cell types (Henson et al., 1992). Calcium levels in neutrophils are not increased evenly throughout the cell during phagocytosis. Calcium is rapidly redistributed within the cell, with the highest concentrations localized near developing phagosomes (Stendahl et al., 1994). It appears that increased Ca^{2+} levels promote clearing of cytoskeletal elements from near the phagosome or plasma membrane, which would allow granules to approach the membrane. Calcium activated annexins then may promote fusion of granule membranes with phagosome and plasma membranes, leading to the discharge of granule contents (Henson et al., 1992).

The granule types differ in their abilities to fuse with the plasma membrane and expel their contents to the exterior of the cell. Generally, secondary granules exocytose more readily and more completely than primary granules (Gennaro et al., 1991; Brown et al., 1991; Bainton, 1992). In human neutrophils, there appears to be a definite order to the ease and completeness of secretion of the four granule types. Secretory vesicles are efficiently mobilized by stimuli that only minimally affect the other granules. The next most easily degranulated type is the gelatinase granule, followed by the secondary granule, then by the primary granule (Sengelov et al., 1993; Sengelov et al., 1995). Primary granules are so reluctant to exocytose that they will do so only if neutrophils are exposed to the cytoskeletal disrupting agent cytochalasin B (Smolen, 1989; Henson et al., 1992). Cytochalasin B inhibits phagocytosis, enhances secretion, and increases the stimulatory effects of many activators of neutrophil function. The mechanism of action of cytochalasin B is poorly understood; it may be

involved in focal removal or dissolution of F-actin from near the plasma or phagosomal membranes, allowing the granules to move close enough to begin the fusion process (Henson et al., 1992).

Differential regulation of degranulation and sequential discharge of the granule types is logical if one considers the physiological role played by neutrophils. Neutrophils in circulation must remain relatively inactive to prevent untimely deployment of their antimicrobial arsenals, which could cause inadvertent injury to normal tissue. However, they must also be able to respond quickly to invasion. In order to effectively deal with bacterial invasion of tissues, neutrophils must rapidly adhere to endothelial cells, migrate through vascular basement membranes and intracellular matrix substances without causing undue injury, and arrive at an area of inflammation with a full complement of microbicidal products. Adhesion molecules and enzymes that dissolve matrix substances are found in the compartments most readily mobilized; degranulation of secretory vesicles, gelatinase granules, and secondary granules allows rapid extravasation, migration, and up-regulation of surface molecules such as complement receptors and Fc receptors. The most potent antimicrobial mechanisms, and the agents most likely to damage normal tissues, are found in primary granules. Primary granules are the slowest to release, so are more likely to be retained until the neutrophils arrive at the site of inflammation. When the neutrophils reach the area where bacteria are present, their surface membranes are freshly stocked with receptors and functional proteins derived from secondary granule (and gelatinase granules and secretory vesicles in human neutrophils)

contents and membranes. Primary granules, which have been held in reserve, can then empty their contents onto the bacteria within the phagosome (Gennaro et al., 1991; Borregaard et al., 1995). Support for this concept comes from observation that the alpha-3 subunit of type IV collagen, which is found in the walls of capillaries, inhibits degranulation by human neutrophil primary and secondary granules (Monboisse et al., 1995). In addition, a protein that is found in human serum, called angiogenin, has recently been shown to inhibit degranulation of neutrophils without blocking chemotaxis, phagocytosis, or the respiratory burst. The angiogenin concentration in plasma of healthy people was found to be high enough to inhibit neutrophil degranulation in vitro (Tschesche et al., 1994).

The sequence of release of the different granule types has not been examined in bovine neutrophils. Studies have shown that secondary granules, which contains adhesion molecules, complement receptors, and matrix-dissolving enzymes, may secrete their contents due to soluble or particulate stimulants in vitro without the need for cytochalasin B (Gennaro et al., 1983a; Burton et al., 1995). Large granules can be induced to exocytose using soluble or particulate stimulants without cytochalasin B, but cytochalasin B greatly enhances the rate and extent of degranulation when particulate stimulants are used (Gennaro et al., 1983a; Zanetti et al., 1990; Zanetti et al., 1991). Exocytosis of primary granules is negligible without cytochalasin B regardless what stimulant is used (Gennaro et al., 1983a; Quade and Roth, 1996). It appears that bovine neutrophil primary and secondary granules may follow an exocytosis sequence similar to their counterparts in human neutrophils.

Stimulants of degranulation

A number of soluble and particulate stimulants have been used to induce exocytosis of neutrophil granules. Opsonized particles, immune complexes, chemotactic factors, phorbol myristate acetate (PMA), the lectin concanavalin A (con A), and various cytokines have been used to stimulate degranulation (Smolen, 1989). The effects of some of these stimulants are mediated by cell surface receptors. Opsonized particles (complement components C3b and C3bi), complement component C5a, leukotriene B_4 (LTB_4), platelet activating factor (PAF), the synthetic peptide formyl-methyl-leucyl-phenylalanine (FMLP), and interleukin-8 (IL-8) are examples of stimulants of degranulation for which neutrophils have membrane receptors (Smolen, 1989; Smith et al., 1992). Other stimulants bypass surface receptors and directly or indirectly activate intracellular enzymes involved in the regulation of degranulation (Smolen, 1989; Watson et al., 1995a). The calcium ionophores ionomycin and A23187, as well as PMA and con A, belong to this group of stimulants. For example, PMA directly activates protein kinase C, which is involved in intracellular signal transduction.

The ability of some of these stimulants to induce degranulation varies between species (Roth, 1994) and between granule types (Gennaro et al., 1991; Henson et al., 1992). For example, FMLP is one of the commonly used stimulants of human neutrophil activity. However, cattle neutrophils are refractile to stimulation by FMLP because they have no receptors for it (Brown and Roth, 1991). Phorbol myristate

acetate is able to induce exocytosis of human neutrophil primary granules. It does not induce bovine primary granule exocytosis (Brown and Roth, 1991; Quade and Roth, 1996), but will cause secretion of bovine secondary and large granules (Gennaro et al., 1983a).

Some activators of neutrophil function are unable to directly cause degranulation, but can "prime" neutrophils to increase their responsiveness to other stimulants. Some stimulants that cause degranulation when present at high concentrations will cause priming at sub-optimal concentrations. Cytochalasin B, IL-8, granulocyte-macrophage colony-stimulating factor (GM-CSF), tumor necrosis factor-alpha (TNF-alpha), platelet activating factor, and interleukin-6 (IL-6) have been used to prime human neutrophils. It appears that neutrophils can exist in a quiescent, non-responsive state, or in a condition ready to respond to stimuli. Priming does not increase the readiness of already responsive neutrophils, but increases the percentage of responsive neutrophils by recruiting non-responsive cells (Daniels et al., 1994). The mechanism of priming is not well understood. Priming does not involve increasing the number or the affinity of surface receptors, but involves changes in signal transduction. Cytochalasin B has effects at the G protein and protein kinase C levels, whereas the physiological agonists effects occur at or distal to G proteins but prior to protein kinase C (Daniels et al., 1994).

Signal transduction mechanisms involved in degranulation

The signal transduction pathways involved in degranulation have been intensively studied. Much is known, but much remains to be elucidated. The results of current research are confusing and often conflicting. Many of the pathways seem to be interconnected. It is beyond the scope of this manuscript to describe in detail the whole body of available information, but a review of the consensus is in order.

Much research has been done on pathways of degranulation in intact human neutrophils, but very little in bovine neutrophils. However, much of the reported research has been conducted on signaling pathways involved in other neutrophil functions (e.g. chemotaxis or induction of the respiratory burst), or has used permeabilized neutrophils, granulocyte cell lines, or other exocytic cell types. Therefore, some of the signal pathways described may not be relevant to the regulation of degranulation following physiological stimuli in normal human or bovine neutrophils.

The first steps in the signal transduction pathway are initiated by receptor-ligand interactions. Binding of a ligand to its receptor causes conformational changes of the receptor protein, leading to activation of heterotrimeric guanine nucleotide-binding proteins (G proteins) in or near the cell membrane. Activated G proteins activate phospholipase C, which cleaves phosphatidylinositol diphosphate, resulting in the formation of diacylglycerol (DAG) and inositol triphosphate (IP_3). IP_3 mediates an increase in cytoplasmic calcium by releasing calcium from intracellular stores and

by allowing the influx of extracellular calcium. Diacylglycerol, together with increased calcium or calcium-calmodulin complex, activates protein kinase C. Protein kinase C then phosphorylates other proteins that mediate functional changes (Jain, 1993). Protein phosphorylation is important in the regulation of degranulation (Burt et al., 1994; Niessen et al., 1994; Walzog et al., 1994).

Increasing cytosolic Ca^{2+} and activation of protein kinase C appear to be essential for degranulation of all granule types (Henson et al., 1992; Baggiolini, 1995). These two events may be sufficient for secretion of the easily mobilized secretory vesicles in human neutrophils, but additional signals are needed to cause degranulation of the other granule types (Borregaard et al., 1995). The intracellular messages that control differential degranulation are poorly understood. Higher levels of free Ca^{2+} are needed to induce release of the other granule types compared to the Ca^{2+} levels required by the secretory vesicles. The intermediate enzymes that are affected by these different Ca^{2+} levels and the non-calcium dependent components of the divergent signaling pathways are only slowly being discovered (Borregaard et al., 1995).

Several types of G proteins have been described (Henson et al., 1992). G_E proteins mediate exocytosis in mast cells, adrenal chromaffin cells, and probably neutrophils (Gomperts, 1990; Philips et al., 1991). G_s proteins stimulate adenylate cyclase, which leads to increased cyclic AMP levels, which inhibits several types of receptor mediated signals (Mueller et al., 1992; Buhl et al., 1994; Rothman and Orci, 1996) via activation of protein kinase A (Buhl et al., 1994). G_i proteins apparently

are able to stimulate or inhibit various intracellular signals (Buhl et al., 1994), including signals sent by other G proteins (Mueller et al., 1992). G_o and G_q proteins have been described but are poorly characterized (Snyderman and Uhing, 1992). G proteins also play a major role in neutrophil phagocytosis (Kabbur and Jain, 1995a). It has been observed that the mixture of trimeric G protein subtypes present in a granulocytic cell line can change as the cells differentiate (Wu et al., 1993). The heterotrimeric G proteins have been demonstrated to activate a variety of intracellular messengers via adapter proteins known as Grb2 (Fialkow et al., 1994). G proteins activate Ras proteins (which can activate Mek, which in turn activates MAP kinase), PI_3 kinase (which releases PIP_3), phospholipase A_2, phospholipase C, phospholipase D, tyrosine kinases and phosphatases, and serine/threonine kinases and phosphatases (Bokoch, 1995).

G proteins mediate two different pathways leading to the activation of Mek (formerly known as MAP kinase kinase) (Crews and Erikson, 1993; Ferby et al., 1994). One of these pathways activates Mek via Mek kinase, while intermediaries in the other pathway include protein products of the proto-oncogenes *ras* and *raf* (Crews and Erikson, 1993). Activated Mek phosphorylates and activates MAP kinase. MAP kinases are a family of serine/threonine kinases that regulate the phosphorylation and activation of several factors, including phospholipase A, other serine/threonine kinases, and can activate transcription factors in the nucleus (Fialkow et al., 1994; Bokoch, 1995). MAP kinase substrates also include microtubule associated proteins MAP-2 and tau. It has been predicted that MAP kinase plays an important role in

cytoskeletal changes (Buhl et al., 1994; Bokoch, 1995), which may imply a role in degranulation. There are multiple pathways at both protein G-Mek and MAP kinase-substrate levels, which complicate investigation (Crews and Erikson, 1993). The MAP kinase pathway in neutrophils may also be activated by reactive oxygen intermediates produced during the respiratory burst. Oxygen radicals have been shown to activate Mek, which in turn activates MAP kinase (Fialkow et al., 1994).

Another group of GTP-binding proteins has been described. These are the low molecular weight G proteins (LMWGP). Many of these are included in the Ras superfamily. Ras LMWGP are involved in linking different signal transduction pathways (Brom et al., 1993). They have been shown to be involved in control of vesicle trafficking, secretion, and motility in some cell types (Bokoch, 1995; Casey, 1995), but this has not been demonstrated in neutrophils. However, LMWGP have been found associated with the membranes of primary and secondary granules in human neutrophils (Henson et al., 1992). These proteins have not been well characterized, but the two granule types have distinctly different subsets of LMWGP types (Philips et al., 1991). While only speculative at this time, these distinct sets of proteins are uniquely situated to play a role in the differential regulation of the granule types (Philips et al., 1991).

A number of other intracellular messengers have been investigated and deserve mention, although their roles in signaling mechanisms are poorly understood. Only a brief summary of these mediators will be presented here.

Arf proteins are involved in vesicle formation and trafficking in a number of cell types (Casey, 1995; Rothman and Orci, 1996). Arf proteins have been found in human neutrophils, but their intracellular location has not been determined and their function remains speculative (Cockcroft et al., 1994; Bokoch, 1995). Arf activates phospholipase D, which is reported to be involved in secretion of specific granules via production of diradylglycerol (Nakamura et al., 1994; Suchard et al., 1994; L'Heureux et al., 1995).

Phospholipase A_2 (PLA_2) is activated by two agents known to be involved in degranulation, protein kinase C and MAP kinase (Lin et al., 1993; Ferby et al., 1994). PLA_2 is a key molecule in neutrophil production of arachidonic acid in response to extracellular stimuli (Matsuda et al., 1994). PLA_2-induced arachidonic acid and its lipid metabolites are involved in degranulation of secondary granules (Jacobson and Schrier, 1993), and metabolites resulting from 5-lipoxygenase activity on arachidonic acid may be important in degranulation of primary granules (Webb and Roth, 1987). Leukotriene B_4 and platelet activating factor are two products of PLA_2-related metabolism that play important roles in degranulation of neutrophils (Lam and Austen, 1992; Zimmerman et al., 1992). Arachidonic acid, besides being an important substrate for the production of other mediators, can contribute to regulation of degranulation by influencing protein kinase C activation and Ca^{2+} flux (Wright et al., 1994). It can also enhance binding of GTP to G proteins (Abramson and Mills, 1991). PLA_2 is typically described as being Ca^{2+} dependent, but a Ca^{2+}-

independent PLA_2 enzyme or route of activation has been reported (Smith et al., 1992).

Leukotriene B_4 (LTB_4) is one of the products of 5-lipoxygenase action on arachidonic acid. LTB_4 produced within neutrophils may be necessary for the cell to respond to external stimuli (Guidot et al., 1994) and is a potent stimulant of degranulation (Lam and Austen, 1992; Gadaleta et al., 1994). LTB_4 is produced in large amounts following stimulation of neutrophils by calcium ionophore or opsonized zymosan (McDonald et al., 1994) and may be necessary for exocytosis of primary granules (O'Flaherty et al., 1990). Exogenous LTB_4 can induce degranulation of primary and secondary granules by binding to G protein-linked surface receptors (O'Flaherty et al., 1990; Lam et al., 1992). LTB_4 appears to work in part by activation of PKC and increased Ca^{2+} levels (Lam and Austen, 1992; McDonald et al., 1994).

Phospholipase C (PLC) has been shown to play a major role in phagocytosis (Kabbur and Jain, 1995a) and exocytosis of primary and secondary granules (Smith and Waite, 1992) in response to some stimuli. There may be distinct subsets of PLC (Wu et al., 1993; Walzog et al., 1994). G proteins can activate PLC (Bokoch, 1995).

Long-chain acyl coenzyme A seems to be involved in degranulation of primary granules, vesicle trafficking, and other neutrophil functions. It also appears to modulate activation of PKC as well as trimeric G proteins and LMWGPs (Korchak et al., 1994).

Cell surface integrin molecules appear to be involved in regulation of degranulation (Clark and Brugge, 1995; Yan et al., 1995) in addition to their better-known roles in neutrophil adhesion and motility. Cross-linking of CD18 molecules on human neutrophils can raise Ca^{2+} concentrations and induce exocytosis of primary granules (Walzog et al., 1994). Adherent neutrophils may respond to stimulants and degranulate more readily than neutrophils in suspension (Richter, 1992). Integrins are linked to actin filaments in the cytoskeleton by several structural proteins, including talin, alpha-actinin, and vinculin. Assembly of these proteins is believed to serve as a framework for the association of various other proteins involved in signal transduction leading to integrin-induced cell functions. Binding of integrins to their ligands can activate PKC, MAP kinase, and PLA_2, increase intracellular Ca^{2+} and arachidonic acid levels, and may enhance the effects of other signals involved in neutrophil secretion (Clark and Brugge, 1995). Activation of PLA_2 can upregulate surface expression of the integrin CD11/CD18 by enhancing specific granule release (Jacobson and Schrier, 1993).

Suppression of degranulation

A number of agents have been shown to suppress degranulation of neutrophils, including hormones, drugs, viruses, and bacteria. Inhibition of neutrophil discharge of granule contents and reactive oxygen species is an area of active research.

Neutrophils have been implicated as the cause of inappropriate tissue injury in some human disease syndromes, such as chronic inflammatory bowel disease and post-infarction reperfusion injury to cardiac muscle. Inappropriate degranulation of neutrophils may contribute to tissue injury in lungs of cattle infected with *Pasteurella haemolytica* (Czuprynski et al., 1991). Many drugs have been investigated for their potential use in therapeutic inhibition of neutrophils. Other compounds have been studied as suppressors of specific steps in signal transduction to help gain knowledge of intracellular communication.

Steroid hormones, both endogenous and exogenous, are potent inhibitors of many aspects of immune function, including several functions of bovine neutrophils. Stress due to shipping, calving, weaning, etc. can lead to release of adrenal corticotropic hormone (ACTH), which raises blood cortisol levels. Increased cortisol levels can impair many neutrophil functions, including degranulation (Roth, 1984; Roth and Flaming, 1990). Exogenously administered ACTH can have the same suppressive effects as physiologically induced cortisol (Roth et al., 1982b; Roth, 1985). Injection of a synthetic corticosteroid, dexamethasone, has suppressive effects on bovine neutrophils (Roth and Kaeberle, 1981b; Roth and Kaeberle, 1982; Webb and Roth, 1987), and has been used as a model of stress-induced immunosuppression in cattle (Roth and Flaming, 1990). Neutrophil suppression due to dexamethasone is more pronounced than that due to cortisol. Normal physiological changes in endogenous steroidal hormones such as estradiol and progesterone or injections of these steroids can inhibit normal functions of neutrophils (Roth et al., 1982a; Roth et

al., 1983). Besides inhibiting degranulation, steroids also suppress neutrophil chemotaxis, respiratory burst, phagocytosis, and adhesion, and may alter the maturation of immature neutrophils (Roth et al., 1981a; Roth and Kaeberle, 1982; Goldstein et al., 1992).

The effects of steroids on neutrophils in vivo may not be due to the direct action of these compounds on neutrophils. Glucocorticoid receptors have been demonstrated in human neutrophils (Schleimer et al., 1989). However, isolated human neutrophils exposed to glucocorticoids in vitro showed no inhibition of degranulation, leukotriene B_4 formation, chemotaxis, or binding to vascular endothelium (Schleimer et al., 1989). It has been reported that incubating cattle neutrophils directly with physiologic or pharmacologic doses of hydrocortisone in vitro did not inhibit iodination, ingestion of opsonized bacteria, or antibody-dependent cell-mediated cytotoxicity (Frank and Roth, 1986).

Glucocorticoids affect other cells of the immune system as well, inhibiting cytokine and growth factor production by lymphocytes and monocytes. Production of many cytokines is reduced, including some that are important to neutrophil function such as granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin-1 (IL-1), tumor necrosis factor-alpha (TNF-alpha), IL-8, and interferon-gamma (IFN-gamma) (Roth and Kaeberle, 1982; Goldstein et al., 1992). Physiologic levels of cortisol may cause lysis of activated, but not resting, lymphocytes (Goldstein et al., 1992). Glucocorticoids may inhibit neutrophils indirectly by suppressing cytokine production. Alternatively, steroids may induce other cell types to produce neutrophil-

inhibiting substances; bovine monocytes exposed to hydrocortisone in vitro have been shown to synthesize proteins that inhibit some neutrophil functions (Frank and Roth, 1986).

Some aspects of the mechanism of action of steroids are well known. Glucocorticoids bind to intracellular receptors. The steroid-receptor complex then moves into the nucleus and binds to glucocorticoid response elements on the DNA. Polymerase can then bind to DNA and begin synthesis of a protein called lipocortin. One action of lipocortin is to suppress the action of PLA_2. This reduces the amount of available arachidonic acid. Lipocortin also inhibits 5-lipoxygenase and cyclo-oxygenase, which further reduces the production of active metabolites of arachidonic acid, including LTB_4 (Webb and Roth, 1987; Goldstein et al., 1992), which is important in degranulation (Lam and Austen, 1992). Steroids also affect cAMP-dependent protein kinases, raise intracellular cAMP levels, and reduce production of certain proteins (Goldstein et al., 1992).

Phenothiazines are a class of drugs used to treat psychosis in humans. They inhibit exocytosis in several cell types, including neutrophils. They have been shown to bind to and inactivate calmodulin, preventing the Ca^{2+} flux needed for exocytosis. They also interfere with membrane fusion events mediated by the annexin proteins (Blackwood and Hessler, 1995).

Viral infections have been implicated in suppression of neutrophil functions, including degranulation. It is believed that viral inhibition of neutrophil function may increase the susceptibility of the host to secondary bacterial infections (Filion et al.,

1983; Abramson and Mills, 1988). Some inhibition may be due directly to infection of neutrophils by the virus. However, neutrophils seem unlikely targets for viral invasion due to their typically low rates of translation and transcription. Viral infection of other leukocytes, e.g. T lymphocytes, may reduce the production of factors that mediate neutrophil activities, thereby indirectly suppressing neutrophils (Atluru et al., 1992; Hartshorn et al., 1992; Palumbo et al., 1994). In general, viral effects on neutrophils are poorly understood. This manuscript will focus on two viruses whose interactions with neutrophils are relatively well characterized; influenza virus in humans and bovine viral diarrhea (BVD) virus of cattle.

Influenza virus inhibits several functions of human neutrophils, including degranulation, chemotaxis, adherence to endothelium, and the respiratory burst (Moore and Mills, 1987; Abramson and Mills, 1988; Hartshorn et al., 1992). Degranulation is inhibited because fusion of primary and secondary granules with the phagosome is disrupted. The reduction in adherence to endothelium is secondary to inhibited fusion of secondary granules to plasma membranes, resulting in reduced surface expression of pre-formed adhesion molecules found in the granule membrane (Moore and Mills, 1987).

Influenza virus infection of neutrophils causes a broad range of functional and apparent metabolic disturbances. Since many of the affected functions involve cytoskeletal rearrangements, it has been speculated that influenza virus directly affects the cytoskeleton or alters intracellular signaling necessary for cytoskeletal function (Abramson and Mills, 1988). Other researchers have demonstrated that

influenza virus interferes with Ca^{2+} mobilization in response to soluble and particulate stimuli, suppresses protein phosphorylation, and may alter protein kinase C activation (Abramson and Mills, 1988; Hartshorn et al., 1988). The current evidence indicates that influenza virus directly affects human neutrophils by interfering with signal transduction, perhaps at the level of receptor-G protein interaction, but more likely at an intracellular location beyond the initial signal (Kazhdan et al., 1994).

Bovine viral diarrhea virus (BVD) is a pathogen of cattle that is found world-wide. It may be the most economically damaging bovine virus in the United States. It causes or contributes to enteritis, respiratory disease, and reproductive losses (Baker, 1995). In addition to the direct effects of BVD virus infection on cattle, BVD virus is immunosuppressive. It causes leukopenia, which may in itself reduce the immune response, but also suppresses functions of lymphocytes (Atluru et al., 1990; Brown et al., 1991), monocytes (Atluru et al., 1992; Welsh et al., 1995), and neutrophils (Roth et al., 1981; Roth and Kaeberle, 1983; Brown et al., 1991). BVD has been shown to inhibit several neutrophil functions, including iodination, intracellular Ca^{2+} flux, migration, the respiratory burst, and antibody-dependent cell-mediated cytotoxicity (Roth et al., 1981; Brown et al., 1991). Even vaccine strains of BVD can suppress neutrophil function (Roth and Kaeberle, 1983). Neutrophils themselves appear to be only minimally infected by the virus (Bolin, personal communication). The addition of recombinant bovine interferon gamma (IFN-gamma) can significantly improve some of the depressed functions of neutrophils

(Brown et al., 1991). Interferon-gamma is a T cell derived cytokine that affects neutrophil function by binding to specific receptors on the cell surface. The mechanism of action of IFN-gamma on neutrophils is not well understood, and seems to involve several possible pathways (Steinbeck et al., 1989; Worku et al., 1994). One important action of IFN-gamma on neutrophils is the priming of neutrophils to enhance their responsiveness to other stimuli (Baggiolini et al., 1992).

Macrophage functions are also inhibited by BVD virus, although macrophages themselves are only minimally infected with the virus (Welsh et al., 1995). It has been suggested that BVD suppression of macrophages may be due to cytokine imbalances caused by viral infection of T cells (Atluru et al., 1992; Welsh et al., 1995). Cytokine imbalances could also contribute to neutrophil suppression. Others have speculated that BVD may interfere with neutrophil intracellular signaling or reduce surface expression of receptor molecules (Brown et al., 1991). A reduction in available IFN gamma due to T cell dysfunction or reduced surface expression of IFN receptors could reduce the number of primed neutrophils, resulting in the suppression of neutrophil functions measured by in vitro assays.

Several types of bacteria or bacterial products have been shown to inhibit neutrophil functions. The capsule of type A *Pasteurella multocida* can inhibit phagocytosis and iodination in bovine neutrophils (Ryu et al., 1984). *Pasteurella haemolytica* produces a leukotoxin that at high concentrations is inhibitory or even lethal to bovine neutrophils (Roth, 1988b; Czuprynski et al., 1991). At low concentrations, the leukotoxin may actually stimulate neutrophils to release toxic

oxygen compounds and discharge granule contents inappropriately, exaggerating the inflammatory response and causing excessive tissue damage (Czuprynski et al., 1991). *Mycoplasma* species can adhere to and inhibit neutrophil functions, probably in part by interfering with signal transduction and Ca^{2+} flux (Thomas et al., 1991; Debey et al., 1993).

Perhaps the most well characterized mechanism of bacterial suppression of neutrophil function is the effect of *Brucella abortus* and *Haemophilus somnus* on bovine neutrophils. These organisms were reported to release substances into their environment that inhibited neutrophil iodination (Chiang et al., 1986; Hubbard et al., 1986). The inhibitory products were identified as GMP and adenine (Canning et al., 1985; Canning et al., 1986; Chiang et al., 1986). Subsequently it was demonstrated that bacterial GMP and adenine preferentially inhibited fusion of primary granules with phagosomes (Bertram et al., 1986). In addition, adenine and GMP have also been shown to inhibit primary granule exocytosis in a dose-dependent manner (Quade and Roth, 1996). The precise mechanism of inhibition has not been determined.

These findings have been interpreted to mean that when a neutrophil recognizes nucleotide fragments, it means that ingested bacteria have been lysed, and the neutrophil stops degranulating in order to conserve its limited supply of antimicrobial substances (Roth, 1988a). In light of recent findings concerning the importance of adenine and guanine compounds in intracellular signal transduction, it may be that the nucleotides released by the bacteria directly interfere with cellular

signaling. Alternatively, the effects of adenine and GMP may be receptor-mediated. Human and canine neutrophils have two types of surface receptors for adenosine (Richter, 1992; Bullough et al., 1995). The A_1 receptor inhibits cAMP formation, and the A_2 receptor stimulates cAMP production. It has been shown that increased cAMP can inhibit transduction of some intracellular signals (Buhl et al., 1994; Rothman and Orci, 1996). Adenosine and adenosine analogues that bind to the A_2 receptor have been shown to suppress neutrophil granule discharge by raising cAMP levels (Mueller et al., 1992; Richter, 1992). In dog neutrophils, exogenous adenosine binding to A_2 receptors can reduce neutrophil adhesion and injury to target cells in vitro by increasing cAMP (Bullough et al., 1995).

Enhancement of degranulation

Since inhibition of neutrophil function appears to be a common problem in stressed or sick cattle, many people have searched for ways to prevent or reverse this inhibition. Bovine respiratory disease complex and mastitis have been used as disease models for this research. Numerous drugs and bacteria-derived preparations have been investigated as immunomodulators or enhancers of neutrophil function; few products have demonstrated repeatable efficacy.

Levamasol and thiabendazole are commonly used cattle parasiticides that have been reported to enhance immune functions, but no consistent benefit to neutrophil

functions could be demonstrated after in vivo administration (Roth, 1984; Roth et al., 1985; Blecha, 1988; Roth et al., 1989; Mulcahy et al., 1991). Avridine is a lipoidal amine that induces interferon production in mice and humans (Kehrli and Roth, 1990). Avridine reversed most of the suppression of neutrophil function seen in dexamethasone treated cattle (Roth and Kaeberle, 1985), but did not affect iodination. Dihydroheptaprenol also induces interferon production, and in cattle it has been shown to increase the number of circulating neutrophils as well as enhance their function (Araki et al., 1987; Yoneyama et al., 1989). Growth hormone, i.e. recombinant bovine somatotropin, is now used commercially in dairy cows to increase milk production. Its effects on the immune system have been studied. Somatotropin generally enhances immune responses (Goff and Roth, 1991). Treated cows had modest elevations of serum immunoglobulins, but no effects on neutrophils were reported (Burton et al., 1991b; Burton et al., 1991a). The anti-coccidia drug decoquinate was shown to enhance iodination in cattle neutrophils (Roth et al., 1989), but it is unknown whether this enhancement was a direct effect on neutrophils, or was secondary to a reduction of the immunosuppressive effect of coccidia.

Endogenously produced substances have been investigated as immunity enhancers. Cytokines, growth factors, and other products of immune cells can directly induce neutrophil degranulation or can prime neutrophils for increased responsiveness to stimulants. Interleukin-6 (Bank et al., 1995), GM-CSF (Kowanko et al., 1991), granulocyte colony-stimulating factor (G-CSF) (Steinbeck and Roth, 1989), neutrophil activating peptide 2 (NAP-2) and macrophage inflammatory

peptide-2 (MIP-2) (Kurdowska et al., 1994) have all been shown to induce degranulation in human neutrophils. Granulocyte-colony stimulating factor (Sordillo et al., 1992) and GM-CSF (Kehrli et al., 1991) can induce degranulation in bovine neutrophils. Interleukin-8 (IL-8) can induce degranulation of primary and specific granules in human neutrophils (Smith et al., 1992; Kurdowska et al., 1994). Recombinant human IL-8 is chemotactic for bovine neutrophils (Persson et al., 1993), but its effect on other bovine neutrophil functions has not been reported. An IL-8-like peptide has been recently characterized in the bovine (Hassfurther et al., 1994). Tumor necrosis factor-beta and TNF-alpha have been reported to cause degranulation in human neutrophils but not in bovine neutrophils (Ferrante et al., 1988; Chiang et al., 1991). Others have found that recombinant human TNF-alpha can induce degranulation of both primary and secondary granules in cattle neutrophils (Sample and Czuprynski, 1991). Interleukin-1 has many effects, including the induction of fever and acute phase proteins, the induction of other cytokines and growth factors (Steinbeck and Roth, 1989a; Canning and Neil, 1989). It has been reported to directly induce degranulation in bovine and human neutrophils (Sample and Czuprynski, 1991; Coyle et al., 1992). Cytokines may be synergistic in their effects on neutrophils. Interferon-gamma, IL-1, and TNF-alpha can all interact and enhance the priming effects of each other (Sample and Czuprynski, 1991). Cytokine mediated events in neutrophils are complicated, interrelated, and involve a number of different enzymes (Kabbur et al., 1995).

Interferon-gamma (IFN-gamma) is a cytokine produced by T lymphocytes and natural killer cells. It has a wide array of effects on various tissues (Abbas et al., 1994). The effects of interferon-gamma on bovine neutrophils have been studied more extensively than the effects of the other cytokines. There are conflicting reports regarding the ability of IFN-gamma to directly cause degranulation in bovine neutrophils. Some have observed that IFN-gamma caused no release of primary or secondary granule contents (Sample and Czuprynski, 1991; Tennenberg et al., 1993), while others have reported that IFN-gamma can cause a weak release of the secondary granule (Steinbeck and Roth, 1989). IFN-gamma can prime neutrophils and enhance their responses to other stimulants. Elastase release from primary granules and iodination caused by stimulation with opsonized zymosan were enhanced in healthy cattle injected with IFN-gamma (Roth and Frank, 1989; Steinbeck and Roth, 1989; Brown et al., 1991).

Priming of neutrophils by IFN-gamma can be even more pronounced in cattle that are immunosuppressed than in normal animals. Neutrophils from cattle treated with dexamethasone or persistently infected with BVD virus show inhibition of several functions, including iodination. Interferon-gamma enhanced responses of neutrophils from these immunosuppressed cattle more than it enhanced responses of neutrophils from healthy animals (Roth and Frank, 1989; Brown et al., 1991). Interferon-gamma prevented suppression of neutrophil degranulation caused by *Brucella abortus*, GMP, and adenine when the cytokine was used with neutrophils in vitro or given to cattle in vivo (Canning et al., 1989). Bovine neutrophils that are

recovered from milk have reduced functions compared to blood neutrophils. In vitro exposure to IFN-gamma reversed the suppression (Sordillo and Babiuk, 1991). It seems that interferon-gamma can enhance the activities of suppressed neutrophils more than it enhances normal neutrophils (Steinbeck and Roth, 1989).

The signal transduction pathway of INF-gamma has been studied primarily in human and rodent cells. Many cell types, including neutrophils, have dimeric receptors on their surface for IFN-gamma. Cytokine binding to these receptors leads to internalization of the IFN-receptor complex. This facilitates binding and activation of JAK1 and JAK2 tyrosine kinases, which initiate the signal transduction cascade probably via the Stat protein, leading to the promotion of transcription of appropriate genes (Oppenheim, 1994; Szente et al., 1995). Receptor activation also leads to activation of phospholipase C and D, with production of diacylglycerol and activation of protein kinase C (Nathan, 1992). In bovine neutrophils, it has been observed that IFN-gamma consistently enhances arachidonic acid metabolism, LTB_4 production, and protein synthesis following stimulation with opsonized zymosan (Steinbeck et al., 1989).

Interleukin-8 is a protein produced primarily by monocytes, although many cell types can produce it when stimulated by IL-1 or TNF (Baggiolini et al., 1992). Interleukin-8 stimulates a number of neutrophil functions, including degranulation of all four granules types in human neutrophils. Cytochalasin B greatly enhances its effect on primary granules (Baggiolini et al., 1992). Receptor binding by IL-8 increases Ca^{2+} levels and activates two subsets of PKC (Smith et al., 1992; Wu et al.,

1993). IL-8 does not activate PLA_2, but does activate phospholipase D and can increase LTB_4 production by neutrophils (Smith et al., 1992; L'Heureux et al., 1995). Recent work with cattle neutrophils has shown that they respond similarly to human neutrophils when exposed to recombinant human IL-8 (Hassfurther et al., 1994).

Platelet activating factor is a phospholipid synthesized in neutrophils, endothelial cells, and monocytes. It has biological activity on many tissue types (Zimmerman et al., 1992). Platelet activating factor has been used to induce exocytosis of bovine neutrophil secondary granules (Burton et al., 1995) and all four granule types in human neutrophils (Zimmerman et al., 1992). The addition of cytochalasin B enhances the ability of PAF to cause discharge of human primary and specific granules, and enables PAF to elicit bovine primary granule exocytosis (Quade and Roth, 1996). Surface receptors for PAF are linked to G proteins that stimulate the production of IP_3 and diacylglycerol, leading to Ca^{2+} flux and PKC activation. Receptors for PAF are also linked to PLA_2, causing release of free arachidonic acid and increased LTB_4 and PAF production (Zimmerman et al., 1992). Neutrophil stimulation by PAF leads to activation of MAP kinase by at least two routes, one Ca^{2+} dependent and one Ca^{2+} independent (Ferby et al., 1994).

Functional deficits of neutrophils from neonates

Neutrophils from neonates and the young of several species, including cattle, are functionally deficient in a variety of ways compared to neutrophils from adults animals, which contributes to an increased susceptibility to disease (Lee and Roth, 1992; Zwahlen et al., 1992; Roth, 1994). Calf neutrophils show deficits in iodination, superoxide anion production, Fc receptor expression, ADCC, chemotaxis, and capping of Con A binding sites (Zwahlen et al., 1992; Roth, 1994). These age-related differences in function are not due to differences in Ca^{2+} flux or in protein kinase C activation (Dore et al., 1990; Dore et al., 1992). Relative to adult neutrophils, calf neutrophils have an increased ability to ingest *S. aureus* and increased antibody-independent cell-mediated cytotoxicity (Lee and Roth, 1992). Neutrophils from young and adult cattle respond similarly to IFN-gamma exposure (Lee and Roth, 1992).

Reduced iodination in calf neutrophils could be due to disorders of normal oxidative mechanisms, lack of production or destruction of H_2O_2, interference with the chemical reaction catalyzed by myeloperoxidase, decreased degranulation, or reduced total amount of myeloperoxidase in the primary granules (Roth and Kaeberle, 1981c). The total myeloperoxidase content of calf neutrophils has been reported to be the same as (Hauser et al., 1986) or significantly less than adult neutrophils (Lee and Roth, 1992; Zwahlen et al., 1992; Quade and Roth, 1996). Degranulation of calf neutrophil primary granules is reported to be more complete

and rapid than adult neutrophils when stimulated by opsonized zymosan and cytochalasin B (Quade et al., 1996). It seems that calf neutrophil primary granules contain less myeloperoxidase than adult neutrophils but that they may degranulate more completely. The other possible mechanisms of reduced iodination apparently have not been examined.

Summary

Neutrophils are critical in prevention of bacterial infections. Cytoplasmic granules and their contents are important parts of the antibacterial capability of neutrophils. The granules fuse with phagosomal or plasma membranes and expel their bactericidal substances and other contents into the phagolysosome or to the exterior of the cell. Numerous genetic defects of neutrophil granules or their components have been described. These defects illustrate the importance of normal granule function because most such defects result in an increased incidence of recurrent bacterial infections, and some defects are potentially lethal.

Neutrophils from different species may possess distinct granule types that may contain unique substances. The contents include many types of antimicrobial enzymes, hydrolases, proteinases, and cationic peptides, as well as adhesion molecules, receptor molecules, and matrix dissolving enzymes. Different granule types may respond to stimulants differently. There may be differences between

species regarding how analogous granule types respond to stimuli. There appears to be a hierarchy in the order of mobilization among the different granule types. Granules containing adhesion molecules, receptors, and matrix-dissolving enzymes degranulate more readily than granule types containing bactericidal compounds. This allows neutrophils to extravasate and migrate through tissues and arrive at a site of infection without depleting their antimicrobial contents or causing inadvertent injury to healthy tissue.

Many assays have been described to measure degranulation of the various granule types. Methods exist to measure primary and secondary granule release in several species, gelatinase granules and secretory vesicles of human neutrophils, and large granules of ruminants. These assays have been used to determine the effect of a variety of neutrophil stimulants on the different populations of granules. The effects of soluble and particulate stimulants that directly induce granule discharge and agents that can prime neutrophils for enhanced response to stimulants have been studied. The effects of inhibitors of granule discharge have also been reported. Agents that inhibit or activate specific intracellular components of signal transduction pathways have been used to help decipher the complex interactions involved in signal transduction and control of granule activity.

Reduced neutrophil degranulation may increase susceptibility to bacterial infection. Degranulation may be suppressed by exogenous agents such as drugs or viral and bacterial infections. Degranulation may also be inhibited by endogenous substances, such as stress-induced cortisol release and physiological fluctuations of

steroid hormone levels. Neutrophils from the newborn of many species have reduced capabilities compared to adults. Preventing or reducing inhibition of granule discharge may help reduce illness in people and animals.

Excessive release of granule substances, or discharge at the wrong time or place, may injure normal tissue. Such inappropriate degranulation has been implicated in contributing to several ailments in humans and in causing lung damage in cattle infected with *Pasteurella haemolytica*. Preventing inappropriate granule discharge may be of value in controlling these illnesses.

In order to design therapeutic drugs that influence degranulation, the regulation of degranulation must be understood. Much progress has been made in understanding external stimuli and intracellular signal transduction mechanisms involved in selective degranulation, but the knowledge is far from complete. More research is needed to complete our understanding of how these vital functions are regulated. With sufficient knowledge, it may be possible to design effective drugs to specifically and selectively enhance or inhibit neutrophil degranulation for therapeutic purposes.

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A RAPID, DIRECT ASSAY TO MEASURE DEGRANULATION OF BOVINE NEUTROPHIL PRIMARY GRANULES

A paper submitted to Veterinary Immunology and Immunopathology

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Abstract

Degranulation of bovine neutrophil primary granules has been measured using various methods. This manuscript describes an assay that measures the exocytosis of myeloperoxidase (MPO), using 3,3',5,5' tetramethylbenzidine as a substrate. The assay is direct, rapid, and quantitative. The assay was used to demonstrate that extracellular Ca^{2+} and cytochalasin B are necessary to induce exocytosis of the primary granule when calcium ionophore, opsonized zymosan, or platelet activating factor are used as neutrophil stimulants. Phorbol myristate acetate was ineffective at inducing degranulation. Primary granule degranulation was rapid, with evidence of MPO release by two minutes and peak measurements of MPO activity occurring between 10 and 20 minutes after stimulation. Adenine and guanine monophosphate were shown to inhibit primary granule exocytosis. Calves were shown to have

significantly less total neutrophil MPO relative to adult cattle. However, calf neutrophils showed a significantly higher percent release of MPO due to stimulation by opsonized zymosan than did neutrophils from adult animals. Percent release of MPO did not differ significantly between calves and adults when calcium ionophore was used as a stimulant.

Abbreviations

CaI, calcium ionophore A23187; CTAB, cetyltrimethylammonium bromide; cyto B, cytochalasin B; DMSO, dimethyl sulfoxide; GMP, guanosine monophosphate; HBSS, Hank's Balanced Salt Solution; MPO, myeloperoxidase; OZ, opsonized zymosan; PAF, platelet activating factor; PBS, phosphate buffered saline; PMA, phorbol myristate acetate; PMN, polymorphonuclear leukocyte; PMSF, phenylmethylsulfonyl fluoride; TMB, 3,3',5',5' tetramethylbenzidine dihydrochloride;

Introduction

Neutrophils are a vital first line of defense against bacterial invasion of the host. Granules within the neutrophils contain a variety of antimicrobial substances. Myeloperoxidase (MPO) is an important component of the primary (or azurophilic)

granules of many species, including humans and cattle. It catalyses the myeloperoxidase-H_2O_2-halide antibacterial reaction. Degranulation of the neutrophil primary granule has been measured by the iodination assay (Roth and Kaeberle, 1981). This is an indirect measurement of degranulation. The assay determines the functional status of the myeloperoxidase-H_2O_2-halide antibacterial system, which involves a complex chain of events. The iodination reaction may be affected by disorders of normal oxidative mechanisms, reduced degranulation, reduced myeloperoxidase content of the primary granule, lack of production or destruction of H_2O_2, or interference with the myeloperoxidase catalyzed reaction (Roth and Kaeberle, 1981).

In this study, bovine neutrophil primary granule degranulation was measured by determining the exocytosis of MPO. The assay used is rapid, direct, colorimetric, and can be quantitated with an ELISA reader. A microtiter plate format is used, which allows a large number of samples to be measured simultaneously. In addition, no radioactive materials are used. This assay was adapted for use with bovine neutrophils following a method previously described for measuring MPO exocytosis of human neutrophils (Menegazzi et al., 1992).

Myeloperoxidase is a strongly cationic protein that tends to stick to cell membranes and test tube walls. Assays that measure the MPO activity of cell-free supernatants of degranulating neutrophils tend to underestimate the actual level of the enzyme released. This assay method allows the determination of enzyme activity before separating the cells and debris from the supernatant. Myeloperoxidase that is

adhered to PMN membranes, particulate stimulants, or microtiter plate wells should be measured, thus allowing a more accurate determination. The colorimetric assay is based on MPO-H_2O_2 oxidation of 3,3',5,5' tetramethylbenzidine, which is considered to be the least toxic of the sensitive peroxidase substrates (Suzuki et al., 1983; Menegazzi et al., 1992).

Many substances and infectious agents have been demonstrated to inhibit iodination by cattle neutrophils (Roth, 1994). It has been shown that *Brucella abortus* and *Haemophilus somnus* inhibit the MPO-H_2O_2-halide system by the release of adenine and guanosine monophosphate (GMP) (Canning et al., 1986). In order to compare this assay with the iodination assay, bovine neutrophils were exposed to various levels of adenine and GMP in vitro to test the effect of the nucleotides on MPO secretion.

Neutrophils from young calves have a number of functional deficits relative to neutrophils from adult animals, including reduced iodination (Roth, 1994). Lower total MPO content could account for this reduction, but there is conflicting evidence regarding the total MPO content of neutrophils from young calves vs mature animals (Hauser et al., 1986; Lee and Roth, 1992; Zwahlen et al., 1992; Roth, 1994). The assay described here was used to measure directly the MPO content of PMNs from immature and adult cattle. The kinetics of primary granule exocytosis of calves and adults were also studied.

Materials and Methods

Neutrophil isolation

Polymorphonuclear leukocytes (PMNs) were isolated from fresh, citrated whole blood from healthy adult Holstein cattle or Holstein calves using a previously described technique (Roth and Kaeberle, 1981). Briefly, PMNs were isolated by centrifuging citrated whole blood and discarding the buffy coat. The packed red blood cell fraction, which also contained the PMNs, was subjected to hypotonic flash lysis. PMNs were separated from lysed red blood cells by centrifugation. The PMNs were resuspended in phosphate buffered saline (PBS) solution at a standard concentration of 5×10^7 PMNs per ml. The resulting cell suspensions consisted of 92-100% neutrophils, with 2-6% monocytes and 0-5% eosinophils.

Reagents

A stock solution of tetramethylbenzidine (TMB, Sigma) (25mM in water) was made fresh each day. The stock solution was diluted in water to a final concentration of 2.5mM immediately before use in the experiments. A solution of H_2O_2 (5mM) was prepared fresh daily by diluting 0.56ml 3% H_2O_2 in 99.44ml distilled water. The detergent cetyltrimethylammonium bromide (CTAB)(0.02% in water) was used as the

lysing agent for determining total MPO content of PMNs. Zymosan opsonized with bovine serum (10mg/ml) was prepared as previously described (Roth and Kaeberle, 1981) and stored at -70°C. Cytochalasin B (1mg/ml), PMA (5mM), CaI (1mg/ml), and PMSF (100μg/ml) were dissolved in DMSO as stock solutions and stored at -70°C. Platelet activating factor (100μg/ml) was dissolved in PBS as a stock solution and stored at -70°C. Aliquots of the reagents were diluted in HBSS for each assay. The final concentrations used in the assays, after addition of buffer and resuspended PMNs, were OZ 1.6mg/ml, cyto B 5μg/ml, PMA 50nM, CaI 5μg/ml, PMSF 1μg/ml, and PAF 1μg/ml. Adenine and GMP (Sigma) were dissolved in HBSS at various concentrations made fresh for each trial. The optimum concentrations of cyto B, PAF, and CaI were determined during preliminary trials. Phorbol myristate acetate was used at concentrations demonstrated by others to stimulate neutrophil functions (Zanetti et al., 1990; Brown and Roth, 1991; Zwahlen et al., 1992).

Assay method

The MPO assay using TMB as a substrate is able to measure only extracellular enzyme. Cytochalasin B can be used to enhance secretion of granule contents to the exterior of phagocytic cells in response to various stimuli (Henson et al., 1992). Neutrophil secretion of MPO in response to PAF, OZ, CaI, and PMA, with and

without the presence of 5μg/ml cytochalasin B was determined. Myeloperoxidase release due to cyto B alone was also examined.

All reagents except PMNs were added to wells of a round-bottom 96-well plate (Costar). All wells contained the same volume of reagents. Wells to measure the total MPO content of lysed PMNs contained 125μl of CTAB solution. Wells for test PMNs contained 125μl of HBSS containing various stimulants. Wells for unstimulated PMNs contained 125μl of HBSS only. Total MPO (lysed cells) and background values (unstimulated PMNs) for each animal were determined concurrently with PMNs exposed to the various stimuli. To examine the necessity of extracellular Ca^{2+} and Mg^{2+} for primary granule release, the experiments were conducted as above, except all reagents were diluted in HBSS without Ca^{2+} or Mg^{2+}. All samples were tested in duplicate.

After pre-warming the microtiter plate containing reagents to 39°C, 25μl of the PMN suspension containing 5 x 10^7 PMNs per ml were added to each well. The plate was incubated at 39°C for 0 to 30 minutes. The plate was removed from the incubator and 50μl of pre-warmed 2.5mM TMB was immediately added to each well, followed immediately by 50 μl of pre-warmed 5mM H_2O_2. The color-change reaction was allowed to proceed for 2 minutes. Then 50μl of 2 N sulfuric acid was added to each well. The acid stopped the reaction and changed the blue color of oxidized TMB to a more stable yellow color. The cells and debris were pelleted by centrifugation at 600 x G for 10 minutes, and 200 μl of supernatant from each well

was transferred to a flat-bottom 96-well plate. The optical density (O.D.) in each well was determined at 405nM using an ELISA reader.

To ensure that the extracellular MPO activity was due to degranulation and not subsequent to PMN lysis, the lactate dehydrogenase (LDH) content of cell-free supernatants was measured by established methods (Taffs and Sitkovsky, 1993). Lactate dehydrogenase is a cytoplasmic enzyme which is released when cells are lysed. PMNs were exposed to the various stimulants as described for up to 30 minutes, then they were chilled, centrifuged, and the supernatant was removed for measurement of LDH.

Effect of adenine and GMP on MPO exocytosis

Opsonized zymosan was chosen as the stimulant to study the effect of adenine and GMP on MPO release since it was the stimulant used in a previous study that measured the effect of adenine and GMP on iodination (Bertram et al., 1986; Canning et al., 1986). Adenine and GMP were dissolved in HBSS and added to microtiter plate wells containing HBSS, OZ, and cyto B. The total volume of reagents in each well was 125μl before PMNs were added. The final concentrations of each nucleotide were 20μg/ml, 40μg/ml, 80μg/ml, and 160μg/ml. Control wells contained only buffer and stimulants. After the plate and reagents were warmed to

39°C, 25μl of PMN suspension was added to each well and the plate was incubated for 15 minutes. The MPO activity in each well was determined as described above.

MPO content of PMNs from animals of different ages

The total MPO content of PMNs isolated from healthy Holstein calves 3-5 weeks of age was compared to PMNs of healthy adult Holstein steers. PMNs were isolated and standardized as described. The eosinophil content of the isolated cells was 0-6 %. PMNs were lysed with CTAB, and the O.D. determined as described above. The kinetics of primary granule degranulation also was compared between the two age groups using the method described above.

The total MPO content of PMNs was calculated from the O.D. of lysed PMNs using a standard curve. Aliquots of pooled bovine PMNs (5 x 10^7/ml) with 1mM PMSF were prepared and stored at -70°C for use as standards. Two-fold serial dilutions of these standard PMN suspensions were lysed with CTAB. The O.D. due to the MPO content of these known concentrations of PMNs was compared to O.D. values due to serial dilutions of commercially available MPO (EC 1.11.1.7, Sigma) and a standard curve was calculated. The aliquots of standard PMNs contained 0.34 units of MPO/10^6 cells and were used in subsequent trials as standards to determine the total MPO of test samples.

Calculating MPO activity

To calculate the percent enzyme activity present in test wells, first the optical density (O.D.) value of wells containing unstimulated PMNs (background) was subtracted from the O.D. values for the stimulated cells and from the values for lysed cells from each animal. The resulting corrected O.D. for stimulated PMNs was divided by the corrected O.D. for lysed cells from the same animal to determine the percent of total enzyme activity released.

% release = (O.D. stimulated - O.D. background)/(O.D. lysed - O.D. background) x 100

For the adenine and GMP inhibition study, percent release values for stimulated PMNs in wells containing the nucleotides were divided by values for control stimulated PMNs for each animal, and the mean percent of control MPO release for each level of test nucleotide reported.

% of control MPO release = (%release with nucleotide / %release of control) x 100

Statistics

Data are presented as means. The statistical significance of differences in percent release due to nucleotides, total MPO PMNs from adults and calves, and kinetics of degranulation were examined by analysis of variance using the SAS system (SAS Institute, 1988). The significance of the differences in percent release due to the various stimulants was determined using Dunnet's T test.

Results

Exocytosis of MPO from bovine PMNs exposed to four different stimulants under various conditions is shown in figure 1. PMNs were exposed to CaI, OZ, PAF, and PMA. The effect of each stimulant was measured with and without physiologic levels of extracellular Ca^{2+} or Mg^{2+}, and with and without 5μg/ml cytochalasin B.

Extracellular calcium appears to be necessary for exocytosis of the primary granule. In medium devoid of Ca^{2+}, with or without Mg^{2+}, MPO release due to stimuli with cyto B was insignificant. MPO release due to stimuli in the absence of both calcium and cyto B was negligible (data not shown).

Cytochalasin B appears to be necessary for these stimulants to cause exocytosis of MPO. In medium with calcium but no cyto B, all the stimulants tested were

essentially unable to induce exocytosis. Cyto B alone, with calcium but with no stimulant, caused 11% MPO exocytosis. The addition of CaI (66.3%), PAF (31.8%), and OZ (28.0%) caused a significant increase in percent MPO release compared to cyto B alone. Phorbol myristate acetate (14.0%) did not significantly increase MPO release compared to cyto B alone. All values reported were with PMNs incubated with stimulants for 15 minutes.

Measurements of lactate dehydrogenase activity in supernatants of PMNs exposed to each of the stimulants ranged from 0 - 3%. This indicates that no stimulant caused a significant amount of cell lysis.

The kinetic study of MPO release (figure 2) demonstrated that neutrophil primary granule secretion occurs rapidly in response to stimulation by either OZ, PAF, or CaI in the presence of cytochalasin B. For all three stimulants, values at 6 minutes were near peak release values. For all stimuli the maximum MPO activity was recorded at 10-20 minutes post-stimulation, with CaI + cyto B causing a maximum of 67.8% of total activity to be released, OZ + cyto B causing a 20.5% release, and PAF + cyto B inducing a 32.5% release. The PMNs were obtained from the same animals (N=5) as the total release study above, but the kinetic study was done on a different day, resulting in slightly different values. In several studies, the MPO activity declined at time points beyond 25 minutes post-stimulation. This decline is probably due to degradation of MPO.

The addition of the protease inhibitor PMSF did not affect the amount of MPO detected. In the kinetic study, PMSF did not affect the rate of decline of MPO activity noted in time periods beyond 20 minutes post-stimulation (data not shown). This might indicate that proteases which are susceptible to PMSF inhibition are not responsible for the decline in MPO activity. Perhaps other proteases or some of the strong oxidizing agents produced during the respiratory burst are responsible for the destruction of the enzyme.

Figure 3 shows the results of increasing levels of adenine and GMP on primary granule degranulation. Both nucleotides inhibited MPO release from PMNs relative to controls, which were stimulated PMNs not exposed to the nucleotides. Adenine suppressed MPO in a dose dependent manner. 20μg/ml of adenine decreased the MPO release to 92% of controls, and increases in adenine suppressed MPO release in a nearly linear manner up to a concentration of 160μg/ml, which inhibited enzyme exocytosis to 65% of control values. GMP inhibited MPO release at concentrations up to 80μg/ml, where MPO release was 83.4% of controls. Higher levels of GMP did not cause further decreases in MPO release. For both nucleotides, the inhibition of MPO exocytosis caused by all concentrations of 40ug/ml and higher were statistically significant ($P < 0.05$).

The total amount of MPO in 5 x 10^7 PMNs from calves was 21.25 units, and the total MPO in 5 x 10^7 adult PMNs was 29.7 units (SEM = 0.1 unit). One unit is defined as the amount of MPO that will produce a change in absorbance of 1 per minute at

470nm using guaiacol as the substrate. The difference in total MPO between age groups was significant ($P < 0.01$).

The kinetics of primary granule release in response to OZ + cyto B and to CaI + cyto B for adult cattle and calves is shown in figures 4a and 4b respectively. Both stimulants cause rapid exocytosis of MPO in both age groups, with near-peak values by 6 minutes post-stimulation, and peak values between 10 and 20 minutes. Maximum % release for adults was 30.4% at 10 minutes for OZ + cyto b and 52.0% at 15 minutes for CaI + cyto B. For calves, the peak % MPO release for OZ + cyto B was 53.7% at 10 minutes, and for CaI was 55.8% at 20 minutes. For calcium ionophore, at no time point were the values significantly different between the age groups. However, PMNs from calves responded to opsonized zymosan more vigorously than did PMNs from adults, with significantly higher percent release of enzyme at all time points ($P < 0.01$).

Discussion

The assay described here differs in several ways from the original assay that was used to quantitate MPO in human neutrophils (Menegazzi et al., 1992). Since neutrophils of cattle contain less MPO than do human neutrophils (Rausch and Moore, 1975; Gennaro et al., 1978), a larger number of cells was required for bovine PMNs (1.25×10^6 cattle PMNs vs. 1.5×10^5 human PMNs per test sample.) The number of

human cells used was small enough that centrifugation of the samples was unnecessary; the cells and debris did not alter the O.D. readings. Since more cattle PMNs were used, centrifugation and removal of the supernatant for O.D. determination was needed to avoid interference with the O.D. reading by cells and debris. In this study, a higher concentration of H_2O_2 than previously reported resulted in optimal readings; the assay for human PMN used 1mM, this study used 5mM H_2O_2. In the originally described assay, 10mM sodium azide in 4N acetic acid was used as a stop solution, and the O.D. of the resulting color was determined at 655nM. In this study, 2N sulfuric acid was used as a stop solution. Sulfuric acid causes a change to a more stable color, and the O.D. was measured at 405nM.

Underestimation of MPO activity due to the cationic enzyme sticking to cell membranes and glass or plastic surfaces should be reduced by adding TMB to the test wells without first removing the supernatant from the wells. TMB and H_2O_2 used at the concentrations used here do not appear to disrupt PMNs and add to MPO release; unstimulated PMNs show consistently low MPO activity, and MPO release does not occur in the presence of 25mM H_2O_2 or 25mM TMB (data not shown). Others have demonstrated that TMB does not react with intracellular peroxidases in intact human neutrophils, or neutrophils treated with cell permeabilizing agents such as digitonin (Menegazzi et al., 1992).

Cytochalasin B was necessary to induce bovine PMNs to degranulate extracellularly in response to calcium ionophore, platelet activating factor, and opsonized

zymosan. PMA did not induce significant degranulation with or without cyto B. Cytochalasin B enhances the secretion of neutrophil granules, and can also cause substances that are poor stimuli to be much more effective (Henson et al., 1992). Others have reported that primary granule exocytosis is minimal unless cyto B is used (Smolen, 1989). Neutrophils often are pre-treated with cyto B for 5-10 minutes before being exposed to stimulants. In this study, no significant differences in degranulation were observed whether PMNs were pre-treated with cyto B for 10 minutes prior to adding stimulants, or if cyto B and stimulants were added at the same time. PMNs treated with cytochalasin B alone showed an average increase of 11% in MPO release compared to untreated PMN. The range of MPO release due to cyto B was 1%-31%. Our group has previously observed that neutrophils from cattle have considerable animal to animal variability in their response to cyto B (Brown and Roth, 1991). For the PMNs that did respond to cyto B alone, additional degranulation of MPO due to PMA was insignificant, while additional MPO release due to OZ, CaI, and PAF was significant on average and for each animal.

Our group has previously reported that bovine PMNs release elastase from the primary granule when stimulated by OZ without the presence of cyto B (Brown and Roth, 1991). In this study, bovine PMNs did not release significant amounts of MPO in response to OZ without the addition of cyto B. Elastase and MPO of bovine neutrophils are both found in the primary granule (Rausch and Moore, 1975; Gennaro et al., 1991; Roth, 1994). One would expect that they would be released by the same stimuli.

Cytochalasin B is believed to inhibit phagocytosis and promote exocytosis of the primary granule (Henson et al., 1992). The MPO release assay described here detects only MPO which is released to the exterior of the cell. The elastase release assay described previously probably measures both the elastase that is released extracellularly and elastase released into the phagolysosome. This is a likely explanation for the observations that cyto B is needed to detect MPO release from primary granules, but is not needed to measure elastase release when OZ is the stimulant.

Physiologic levels of Ca^{2+} appear to be necessary for primary granule exocytosis. None of the stimulants tested caused a significant release of MPO when resuspended in medium lacking Ca^{2+}, whether cyto B was present or not. PMNs did not release MPO in medium containing Mg^{2+} but lacking Ca^{2+}.

Adenine and GMP have been shown to inhibit bovine neutrophil iodination. In a previous study, 40ug/ml adenine inhibited iodination to 83% of control, and 40ug/ml GMP inhibited iodination to 62% of control (Canning et al., 1986). In this study, 40ug/ml adenine decreased MPO release to 81.5% of control, and 40ug/ml GMP inhibited MPO release to 89.8% of control PMNs. Inhibition of MPO release increased to 65.7% of control for adenine at 160μg/ml and 83.4% of control for GMP at 80μg/ml. The amount of suppression caused by GMP is somewhat different between the studies, but the effect of adenine is similar. These results support the theory that release of adenine and GMP by *Brucella abortus* and *Haemophilus somnus* may contribute to the virulence of these organisms by inhibiting degranulation of the neutrophil primary

granule (Canning et al., 1985; Bertram et al., 1986; Canning et al., 1986; Chiang et al., 1986).

MPO deficiency is considered as a possible reason for the observed suppression of the iodination assay in neutrophils from calves (Lee and Roth, 1992). However, there have been varying reports regarding the total MPO content of neutrophils from immature and adult cattle (Hauser et al., 1986; Lee and Roth, 1992; Zwahlen et al., 1992). Some of the variation in results may be due to different ages of the immature animals, and to the different MPO assay methods that were used. The results of this study indicate that adult cattle have significantly more MPO than 3-5 week old calves. The difference in total MPO between age groups could not be explained by differences in the percentage of contaminating eosinophils in the isolated cells.

If the timing of degranulation were delayed in calves, the delay could cause less MPO to be delivered to the site of the MPO-H_2O_2-halide reaction during a critical phase, contributing to the observed reduction in iodination. However, this study indicates that percent degranulation due to a particulate stimulant, OZ, is significantly enhanced in calf PMNs compared to adult PMNs. Degranulation due to the soluble stimulant CaI was not significantly different between the age groups. Calf PMN % degranulation was the same for both stimulants, but adults released less MPO in response to OZ than to calcium ionophore. This study indicates that calf PMNs contain less total MPO, but release a higher percentage of their MPO in response to opsonized particles than do PMNs from adults.

Opsonized zymosan and CaI induce degranulation in different ways. Opsonized zymosan binds to complement receptors on the cell surface, initiating a series of intracellular signals that leads to degranulation. Calcium ionophore bypasses cell surface receptors. It allows transport of calcium ions into the cell, thereby directly or indirectly activating calcium-dependent intermediaries, such as protein kinase C, phospholipase C, and phospholipase A_2, which leads to degranulation (Smolen, 1989; Watson et al., 1995). One might deduce that whatever differences there may be in the regulation of primary granules between neutrophils of the different age groups occurs at the level of surface receptors or early in signal transduction, prior to the activation of the above enzymes.

In summary, the described assay is a useful method of measuring neutrophil primary granule degranulation and total MPO content of PMNs. The assay is direct, quantitative, can rapidly measure a large number of samples, and uses no radioactive reagents. It should provide a more accurate measurement of MPO exocytosis than techniques that measure the MPO activity only in the supernatant of degranulating PMNs.

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Captions for figures

Figure 1. The effect of various stimulants on neutrophil MPO exocytosis in the presence or absence of Ca^{2+} and/or 5μg/ml cyto B. The first group of bars represents PMNs with Ca^{2+} and without cyto B. The second group of bars represents PMNs with Ca^{2+} and cyto B. The third group of bars represents PMNs without Ca^{2+} and with cyto B. The concentrations of the stimulants are: 5μg/ml calcium ionophore (CaI), 1.6 mg/ml opsonized zymosan (OZ), 1μg/ml platelet activating factor (PAF), and 5μg/ml phorbol myristate acetate (PMA). PMNs were incubated with the stimulants at 39°C for 15 minutes (n = 8).

Figure 2. The kinetics of MPO release from adult cattle PMNs in response to 1μg/ml PAF, 5μg/ml CaI, and 1.6 mg/ml OZ. PMNs were exposed to 5μg/ml cyto B concurrently with the stimulants (n = 5).

Figure 3. The effect of increasing concentrations of adenine and GMP on adult bovine neutrophil MPO exocytosis induced by OZ with 5μg/ml cyto B. The data shown represent the % MPO release of PMNs exposed to nucleotide compared to control PMNs. The decreases in % MPO release from control values are significant ($P < 0.05$) for all points at 40μg/ml, 80μg/ml, and 160μg/ml (n =8).

Figure 4a. The kinetics of neutrophil MPO release from calves and adults in response to OZ with 5μg/ml cyto B. The differences between the age groups are statistically significant ($P < 0.01$) for all time points ($n = 7$).

Figure 4b. The kinetics of neutrophil MPO release from calves and adults in response to 5μg/ml CaI with 5μg/ml cyto B. At no time point are the differences between the age groups significant ($P > 0.05$) ($n = 7$).

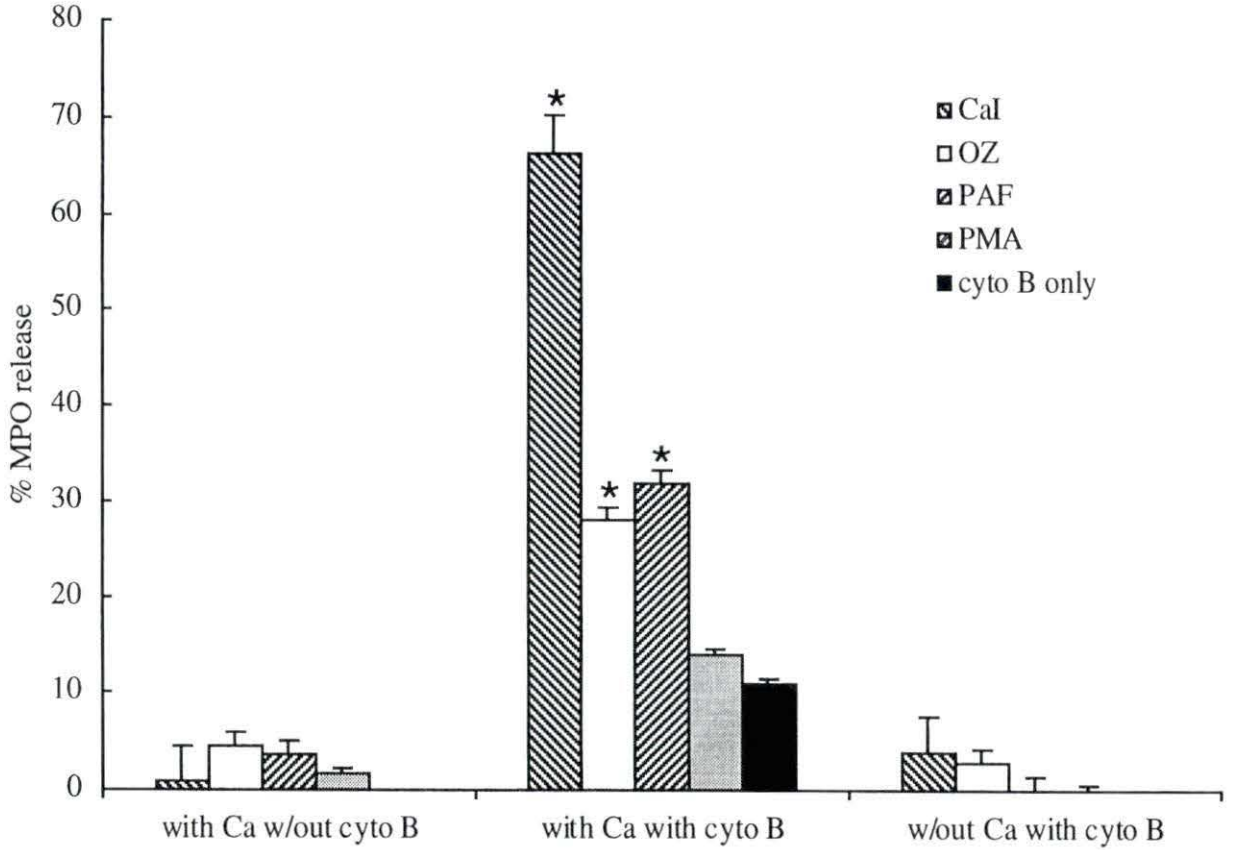
80
70
60
50
40
30
20
10
0
% MPO release
*
*
*
CaI
OZ
PAF
PMA
cyto B only
with Ca w/out cyto B
with Ca with cyto B
w/out Ca with cyto B

Figure 1

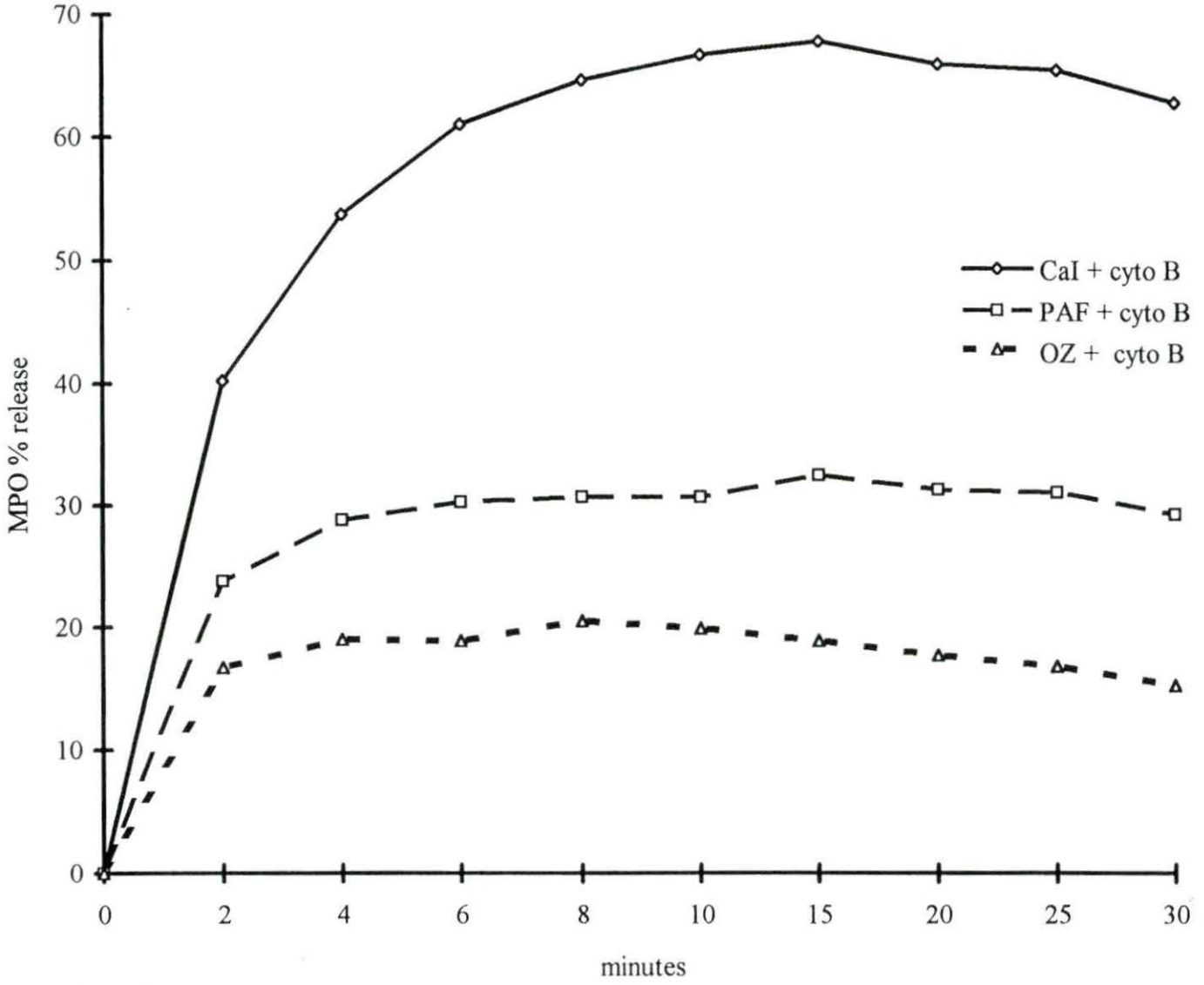
70
60
50
40
30
20
10
0
MPO % release
CaI + cyto B
PAF + cyto B
OZ + cyto B
0
2
4
6
8
10
15
20
25
30
minutes

Figure 2

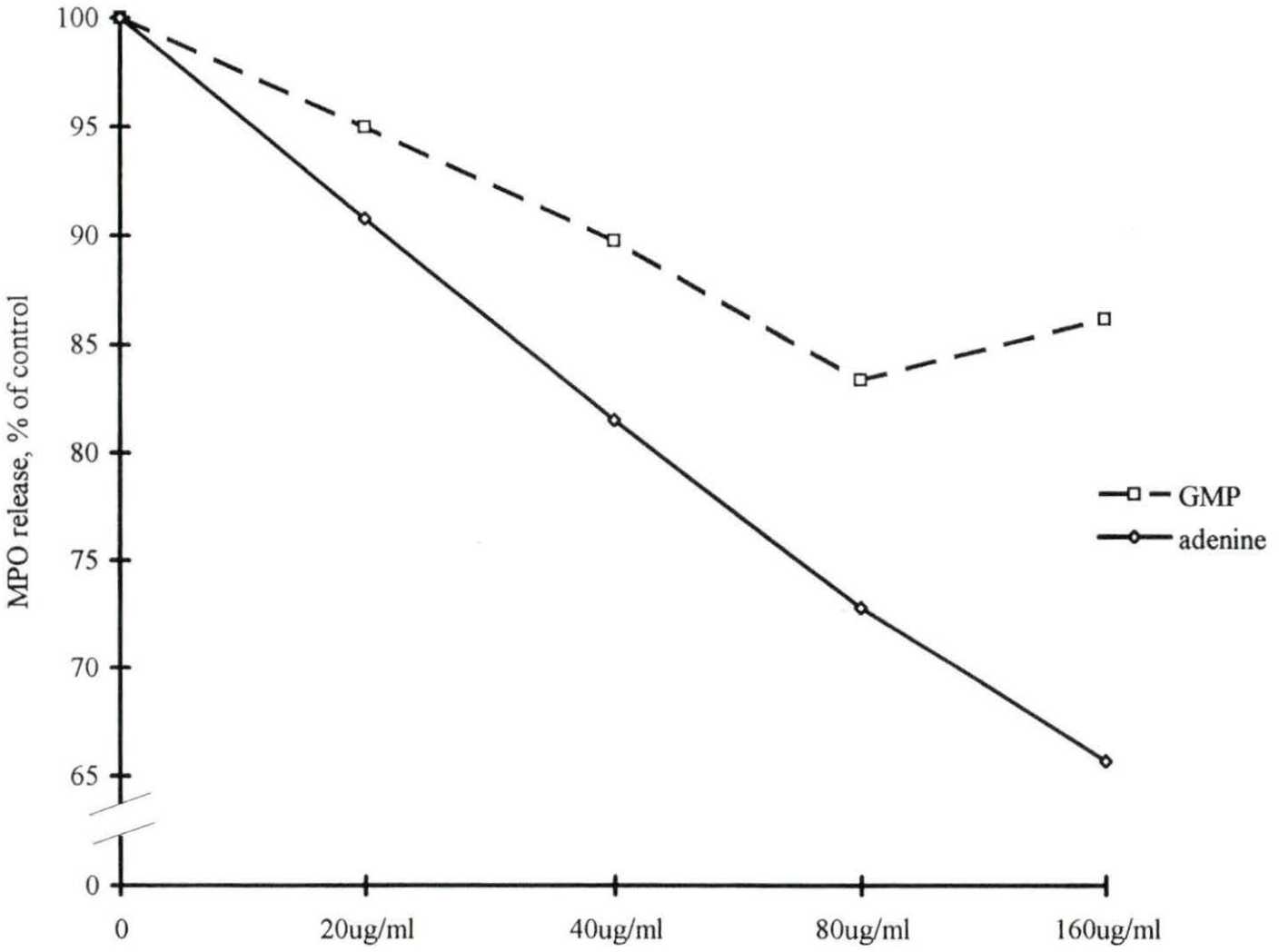
100
95
90
85
80
75
70
65
0
MPO release, % of control
0
20ug/ml
40ug/ml
80ug/ml
160ug/ml
GMP
adenine

Figure 3

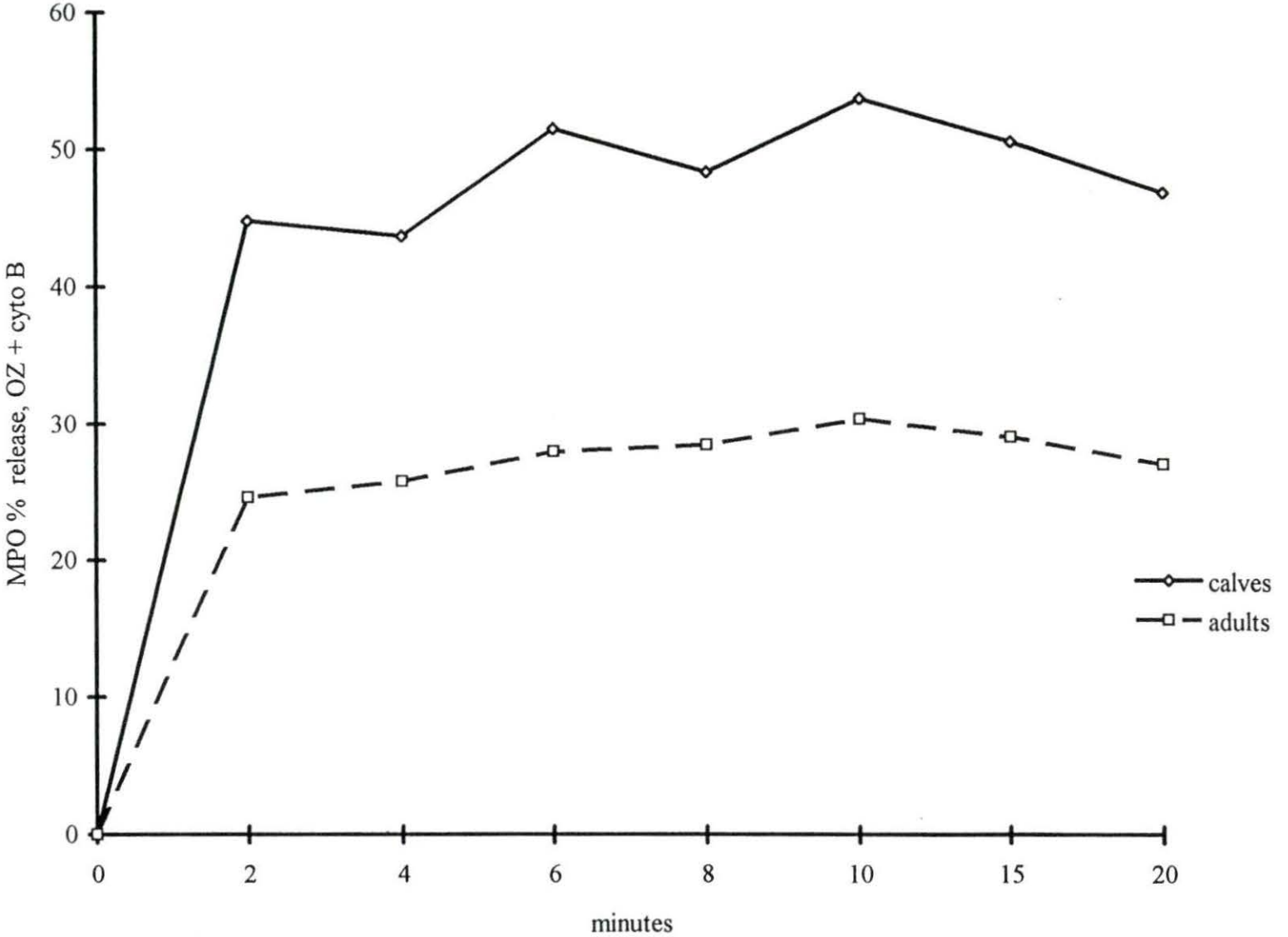
60
50
40
30
20
10
0
MPO % release, OZ + cyto B
0
2
4
6
8
10
15
20
minutes
calves
adults

Figure 4a

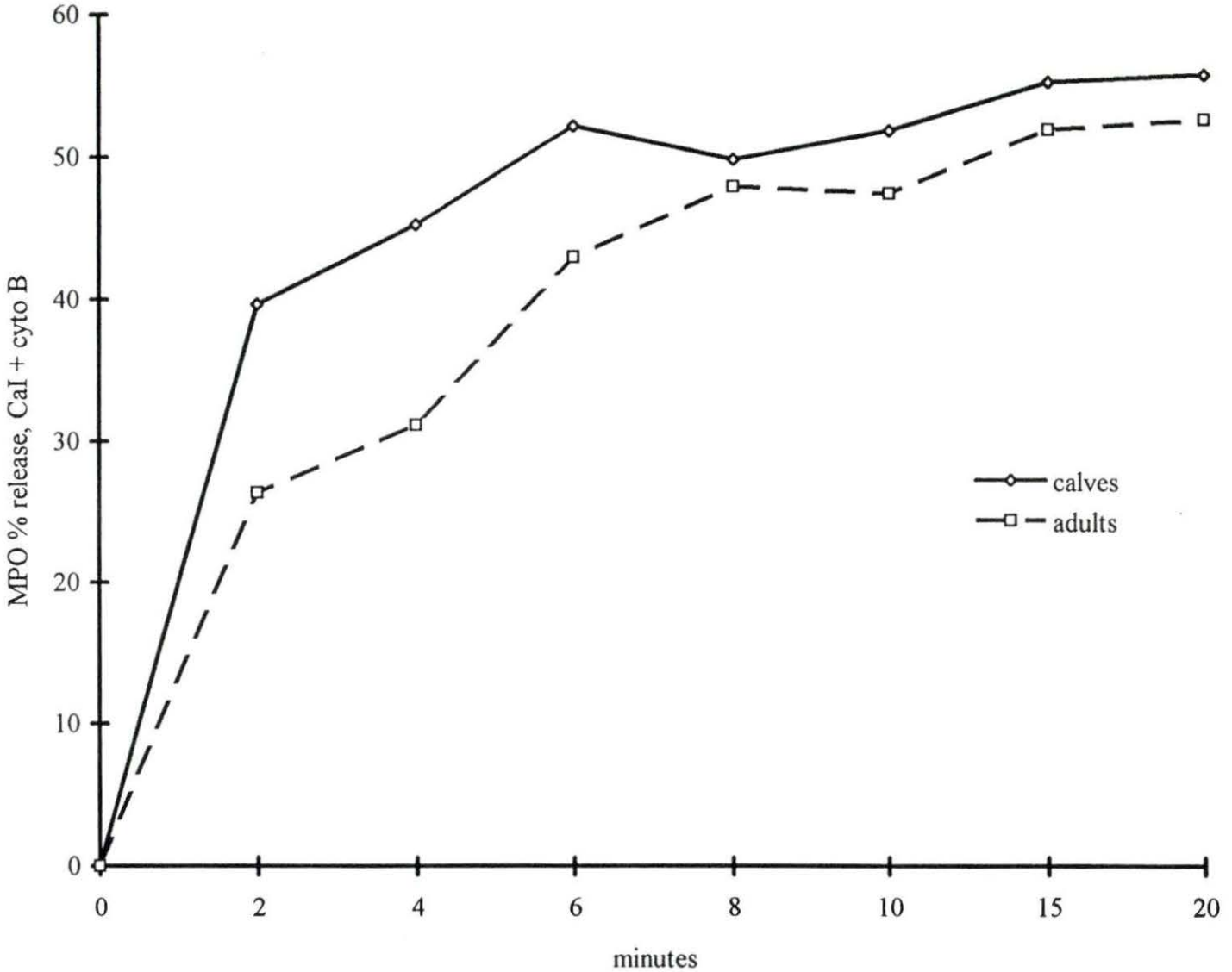
60
50
40
30
20
10
0
MPO % release, CaI + cyto B
0
2
4
6
8
10
15
20
minutes
calves
adults

Figure 4b

ATTEMPTS TO QUANTITATE BOVINE NEUTROPHIL LARGE GRANULE DEGRANULATION

A paper prepared for submission to Veterinary Immunology and Immunopathology

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Introduction

A distinguishing structural characteristic of ruminant neutrophils is the presence of a unique third granule type not found in other species (Gennaro et al., 1983a). Ruminant neutrophils have peroxidase positive granules which form early in granulopoiesis, and peroxidase negative granules of similar size which form late in cell development. These granule types have the characteristics of the primary (azurophilic) and secondary (specific) granules seen in other species. The unique granule population of ruminant neutrophils, called the large granules, is formed after the primary granules but before the secondary granules during cell differentiation. Large granules contain unique oxygen-independent cationic antimicrobial peptides called bactenecins. Large granules also contain lactoferrin, but do not contain peroxidase, lysosomal hydrolases, or other substances found in the other granule types (Baggiolini et al., 1985).

The large granules were first observed in granule fractions of lysed bovine neutrophils subjected to zonal differential sedimentation. In addition to the expected populations of primary and secondary granules, a third granular fraction was noted that did not contain the usual marker enzymes and biochemical identifiers. The granules of this new fraction contained only lactoferrin and antimicrobial substances, which were identified as cationic proteins (Gennaro et al., 1983a). The large granules are larger, denser, and more numerous than the other granule types. Collectively, they form the largest intracellular storage organelle of cattle neutrophils. Large granules are most numerous in the neutrophils of cattle, but are also present in the neutrophils of other ruminants, such as sheep, goats, deer, and ibex. Large granules are not found in neutrophils of humans, horses, or rabbits (Gennaro et al., 1983a; Baggiolini et al., 1985).

The cationic proteins contained in the large granules are able to kill *Staphylococcus aureus* and *Escherichia coli* at very low concentrations. The proteins are most actively microbicidal at pH values near 7.0-7.5, and are not inactivated by physiologic levels of Na^+, K^+, Mg^{2+}, or Ca^{2+}. It is calculated that a discharge of only 10-15% of a neutrophil's content of these proteins into the phagosome would result in bactericidal concentrations of cationic peptides (Gennaro et al., 1983b).

The first of the large granule cationic peptides to be isolated was a cyclic peptide twelve amino acids in length. This peptide is active against *S. aureus* and *E. coli*. The authors called it "bactenecin" from the Latin *bacterium* and *necare*, to kill (Romeo et al., 1988). A monoclonal antibody (Mab) was developed that recognized

this dodecapeptide. This Mab also recognized several other peptides of 4-8 kilodaltons (kD) and 1.6 kD that had antibiotic properties, as well as larger peptides that were not bactericidal. It was speculated that the smaller active peptides might be derived from larger inactive precursors (Marzari et al., 1988).

Two more bactenecins have been described. They have molecular weights of 5 Kd and 7 kD and are called Bac5 and Bac7 respectively. These bactenecins are able to kill a number of Gram-negative pathogens, including *E. coli*, *Klebsiella pneumoniae*, *Salmonella typhimurium* and *Leptospira interrogans*. They arrest the growth of *Enterobacter cloacae* and *Pseudomonas aeruginosa*. They are ineffective against *S. aureus, Streptococcus agalactiae*, *Proteus vulgaris,* and *Borrelia burgdorferi*. The bactenecins also have antiviral properties; they are able to inactivate human herpes simplex virus types 1 and 2 (Zerial et al., 1987). It is estimated that 10^6 cattle neutrophils contain 125ng each of Bac5 and Bac7. These two peptides make up about 4% of the total protein in cattle neutrophils (Zanetti et al., 1991). It is calculated that only about 10% of a neutrophil's Bac5 or Bac7 would need to be discharged into a phagosome to achieve bactericidal concentrations (Gennaro et al., 1989; Scocchi et al., 1993). The bactenecins are believed to be an important component of ruminant defense against bacterial infections (Gennaro et al., 1989).

The sequences of Bac5 and Bac7 have been published (Frank et al., 1990). Bac7 is 59 amino acids in length, and Bac5 consists of 42 amino acids. Both are composed of more than 45% proline and more than 23% arginine, but their sequences are distinct. The structure of the bactenecins has been called "definitely

unusual" because of their low number of component amino acids and the large numbers of proline and arginine residues, and they exhibit little homology with other known protein sequences (Frank et al., 1990). Computer modeling of the sequences suggests that the bactenecins may adopt amphiphilic structures on membrane surfaces (Frank et al., 1990).

Bactenecins are translated from mRNA as pre-probactenecins while developing neutrophils are in the bone marrow. Mature peripheral neutrophils are unable to synthesize bactenecin precursors. The primary transcript of pre-proBac7 is a 23.5 kD polypeptide, and the pre-proBac5 is a 21 kD product. The pre-probactenecins are processed and stored in the large granules as probactenecins of 20 kD (proBac7) and 15 kD (proBac5). The probactenecins are not bactericidal, and must be cleaved by neutral serine proteinases to attain the biologically active form. In bovine neutrophils, serine proteinases are found in primary granules. In order for the mature microbicidal bactenecins to be formed, both the large granules and the primary granules must discharge their contents into the phagosome or to the cell exterior (Zanetti et al., 1990; Zanetti et al., 1991). Elastase is the neutral serine proteinase that is responsible for processing of probactenecins to the active form (Scocchi et al., 1992). Enzymatic cleavage of proBac7 results in formation of the bactericidal 7 kD Bac7 and a small amount of an inactive peptide of 14.5 kD.

The bactericidal mechanism of action of bactenecins has been elucidated (Skerlavaj et al., 1990). Since they are strongly cationic, they are attracted to the negatively charged surface of bacteria. They bind electrostatically to the bacterial

outer membrane and insert into the membrane. Within five minutes, a dramatic increase in permeability of the inner membrane is seen, followed by a suppression of oxygen metabolism by the electron transport chain, reduced transmembrane potential, and inhibition of ATP production. Inhibition of aerobic respiration appears to be a direct effect of bactenecin interaction with some component of electron transport, not merely due to abnormal membrane permeability and the loss of membrane potential. These effects result in reduced synthesis of protein and nucleic acid precursors. Lactoferrin, also found in large granules, apparently works synergistically with the bactenecins, markedly increasing their bactericidal activities (Skerlavaj et al., 1990). Bac5 and Bac7 are most effective at pH 7-7.5, and their effectiveness drops as pH becomes more acidic. The pH within phagosomes normally falls to pH 4-5 after the granules fuse with it and release their contents into it, but bactenecins work fast enough to retain their effectiveness before conditions become too acidic (Skerlavaj et al., 1990).

Staphylococcus aureus and *Streptococcus agalactiae* are resistant to Bac5 and Bac7. Mammalian cells are also resistant to the action of bactenecins. Bac7 inhibits human herpes simplex virus in vitro, but has no cytotoxic effects against the cell lines used to grow the virus. Bac5 and Bac7 do not lyse red blood cells even at concentrations much higher than are sufficient to kill susceptible organisms. Resistance of Gram-positive microbes and mammalian cells may be due to insufficient binding of the cationic peptides to the membranes (Skerlavaj et al., 1990). At neutral pH, Bac5 and Bac7 bind avidly to the surface of *E. coli*. Probactenecins

also bind to *E. coli* at pH 7, but are not bactericidal due to their inability to penetrate bacterial membranes. At acid pH, bactenecins bind to the surface of *E. coli* poorly. In contrast, some of the contents of the primary granule, e.g. myeloperoxidase, bind *E. coli* tightly at lower pH and bind minimally at neutral pH (Litteri and Romeo, 1993).

Large granules have been shown to secrete their contents to the exterior of the cell when neutrophils are stimulated by phorbol myristate acetate (PMA), calcium ionophore A23187, and opsonized particles (Gennaro et al., 1983a; Zanetti et al., 1991). The response of large granules to various stimulants appears to resemble the response of secondary granules more than primary granules (Gennaro et al., 1983a; Zanetti et al., 1991), but the regulation of degranulation of large granules has not been thoroughly explored. There are a number of agents that inhibit or enhance discharge of the other granule types; the effects of such agents on the discharge of large granules has not been reported. The goal of this research was to more closely examine the response of the bovine neutrophil large granule to known stimulants, inhibitors, and enhancers of neutrophil function. It has been proposed that the large granule and its antimicrobial peptides play an important role in native defense against bacterial infection in ruminants (Gennaro et al., 1989). It is our hypothesis that neutrophil-inhibiting agents may interfere with large granule release, thereby increasing susceptibility to bacterial infection. We further hypothesize that such inhibition may be prevented or reversed by some of the enhancers of neutrophil function.

The inability to produce monospecific anti-bactenecin antibody prevented testing of our hypotheses. The sera of the rabbits that were used contained antibodies that recognized several structures of lysed bovine neutrophils. These antibodies were present before the rabbits were immunized. A number of different techniques were used in an attempt to separate the specific anti-bactenecin antibodies from non-specific anti-neutrophil antibodies, but no method resulted in a sufficiently pure antibody preparation. Partly because of the impure antibody, the assay method used to quantify bactenecin discharge from stimulated neutrophils had extreme day-to-day variability. In addition, the assay relies on the ability to determine the total bactenecin content of lysed neutrophils using monospecific antibody to detect the bactenecins. Since the anti-neutrophil antibodies could not be removed, it was impossible to accurately measure the bactenecin content of lysed neutrophils.

Materials and Methods

Producing the synthetic peptide

The published amino acid sequences of Bac5 and Bac7 (Frank et al., 1990) were entered into the PCGENE protein analysis program (Intelligenetics) and McVector software analysis program (International Biotechnologies, New Haven, CT), which were used to predict tertiary structures, flexibility, and the hydrophilicity

of each peptide. Generally, the more hydrophilic a peptide sequence is, the more antigenic it is. For both peptides, the most hydrophilic portion of the molecule is the amino terminal 20 to 25 amino acids. The hydrophilicity index for the amino terminal 20 amino acids of Bac7 was higher than for Bac5, so amino acids 1 - 20 of Bac7 were chosen to synthesize. This sequence was Arg-Arg-Ile-Arg-Pro-Arg-Pro-Pro-Arg-Leu-Pro-Arg-Pro-Arg-Pro-Arg-Pro-Leu-Pro-Phe.

The peptide was synthesized at the Iowa State University Protein Facility using a solid-phase peptide synthesis technique with an insoluble polymeric resin support. It was anticipated that the synthetic peptide would be poorly immunogenic due to its small size. To increase the immunogenicity of the peptide, it was conjugated to a larger protein to make it antigenic as a hapten. The carboxy terminal amino acid of the synthetic protein was changed from phenylalanine to cysteine to facilitate the conjugation procedure.

Hemocyanin from keyhole limpets (KLH) was chosen as the protein to which the synthetic peptide would be linked. KLH has numerous reactive side-chains for coupling peptides, and has proven efficacy (Maloy and Coligan, 1991). Conjugation was carried out using 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide-HCl (EDCI) according to established procedures (Maloy et al., 1991). Briefly, 10mg of synthetic peptide was dissolved in water, 40mg EDCI was added, and the pH of the mixture was adjusted to 4.5 using 0.1M HCl. Ten mg KLH was dissolved in water and added to the peptide-EDCI mixture. Conjugation was carried out by incubating the mixture

of peptides at 4^0C for two hours with stirring. The conjugated protein was then dialyzed against water to remove impurities, aliquoted, and stored at -20^0C.

Animals

Neutrophils were obtained from healthy Holstein steers that were bled by jugular venipuncture. Polymorphonuclear leukocytes (PMN) were isolated using a previously described technique (Roth and Kaeberle, 1981). Briefly, 250 ml of blood was collected into acid-citrate dextrose and centrifuged at 600 x G for 20 minutes. The buffy coat was discarded, and the packed red blood cell fraction, which contains the PMNs, was subjected to hypotonic flash lysis. The PMNs were separated from the lysed red blood cells by centrifugation, and resuspended in phosphate buffered saline (PBS) at a standard concentration of 5 x 10^7 PMNs per ml. Isolated cells consisted of 94-99% neutrophils, 0-6% eosinophils, and 0-3% mononuclear leukocytes.

New Zealand white rabbits were used to produce polyclonal antibody against the synthetic peptide. Two rabbits received an initial immunization with 100ug of synthetic peptide mixed with Hunter's Titermax (CytRx Corp.) adjuvant intramuscularly. Two rabbits received 100ug of conjugated peptide-KLH (bac-KLH) with Titermax intramuscularly. Booster injections of peptide or conjugated protein without adjuvant were given at three and six weeks after the initial injection. A second group of five rabbits was injected with 100ug of bac-KLH with Titermax, with

booster injections without adjuvant given at 4, 8, and 12 weeks. Blood was collected from the rabbits from the central artery of the ear on the day of the first immunization and every three to four weeks thereafter. The blood was allowed to clot, the serum was separated by centrifugation and stored at -20°C in one ml aliquots.

Antibody purification

Rabbit serum was heated to 56°C for 30 minutes to inactivate complement. The immunoglobulin G (IgG) fraction of the serum protein was precipitated from serum by mixing one part saturated sodium sulfate solution to two parts serum and incubating overnight at 4°C. The mixture was centrifuged and the supernatant was discarded. The retained precipitate was resuspended in PBS and dialyzed against PBS at 4°C. The resulting solution was concentrated by centrifugation using a Centriprep (Amicon) with a molecular weight cut-off of 10,000 Daltons. The IgG fraction of the solution was purified by column chromatography using a protein A column (Sigma). The protein A in the column was prepared for use by washing with a series of buffers of decreasing pH, from pH 7 to pH 3. The column matrix was then equilibrated with a pH 7 buffer. The IgG solution was added to the column and incubated at room temperature for two hours. The excess fluid was allowed to flow off the column, but was retained and returned to the column twice. Unbound immunoglobulin was removed by washing the column with 10 column volumes of

pH 7 phosphate buffer. Bound IgG was eluted from the column with 5 volumes of pH 3 citrate buffer. The IgG fractions were immediately restored to neutral pH with 1.0N NaOH and stored at 4°C in aliquots.

As an alternative to protein A chromatography, purification of the anti-bactenecin antibody was attempted using affinity chromatography. Synthetic peptide or bac-KLH conjugated protein dissolved in HEPES buffer was bound to Affigel 10 or Affigel 15 (Biorad) under aqueous conditions for four hours at 4°C. Unbound reactive sites on the Affigel were blocked with 1M ethanolamine. The Affigel with the bound synthetic peptide or bac-KLH conjugate protein was transferred to a column. The gel in the column was washed with a series of citrate buffers of decreasing pH, from pH 7 to pH 3. The column matrix was equilibrated to pH 7 with several column volumes of 50mM HEPES buffer. Immune serum was heated to 56°C for 30 minutes to inactivate complement. The heat inactivated serum was added to the affinity column and incubated at room temperature for 2 hours. Unbound proteins were removed by washing the column with several column volumes of 50mM HEPES solution or PBS at pH 7. Bound immunoglobulins were eluted with a pH 3 citrate buffer. The pH of the eluted fractions was immediately returned to neutrality, and the eluate was aliquoted and stored at -20°C. An alternative elution method was to prepare buffers of progressively lower pH, e.g. pH 6, pH 5, pH 4, and pH 3, to slowly lower the pH of the column. The pH of the resulting fractions was immediately restored to pH 7 and stored as aliquots. Another elution strategy used was to wash the unbound serum proteins from the column with 1M urea in PBS,

followed by elution of the bound immunoglobulin with 8M urea in PBS. The resulting eluent was dialyzed against PBS to remove the urea, and stored in aliquots.

Attempts were made to remove non-bactenecin antibodies from the rabbit serum by adsorption to cell preparations prior to affinity chromatography. Phosphate buffered saline suspensions of whole bovine PMN, whole porcine PMN, and lysed porcine PMN were prepared. Heat inactivated serum was incubated with the various PMN preparations at 4°C for 1 hour. After incubation, the cells and/or debris were removed by centrifugation, and the supernatants retained. Serum samples were incubated with one type of PMN preparation, or were incubated with two or three types of PMN preparations in succession.

Bovine neutrophil preparations

To induce discharge of the large granules, PMNs (5×10^7) in 1 ml PBS or Hanks Balanced Salt Solution (HBSS) were incubated with zymosan opsonized with bovine serum (OZ), 10-100nM PMA, or 1-10ug/ml calcium ionophore A23187 at 37°C for 15-60minutes. PMNs were exposed to these stimulants with and without concurrent exposure to 5ug/ml cytochalasin B. To determine the total content of bactenecin, 5×10^7 PMNs were lysed by resuspending in one ml of 2% Triton X-100 in water, or were resuspended in PBS and lysed by sonication.

After incubation with stimulants or after lysis, PMN preparations were centrifuged briefly to pellet cells and debris, and the cell-free supernatant retained to

be used directly in western blots or in quantitative dot blots. Alternatively, the supernatants were treated with 10% trichloroacetic acid (TCA) to precipitate the proteins. The precipitate was pelleted by centrifugation at 1500 x G for 10 minutes. The pelleted proteins were then resuspended in PBS or in western blot sample buffer.

The effects of pH and a proteinase inhibitor on maturation of the bactenecins were also examined. Bovine PMNs were isolated as above and resuspended in pH 7 PBS, pH 7 PBS containing the serine proteinase inhibitor phenyl-methyl-sulfonyl-fluoride (PMSF) 1mM, or pH 4 citrate buffered isotonic saline solution. The cells were then lysed by sonication. Proteins in the cell lysates were precipitated with 10% trichloroacetic acid (TCA), pelleted by centrifugation, and resuspended in PBS or in western blot sample buffer.

Western blotting technique

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to a previously described procedure (Laemmli, 1970). Separating gels of 10-20% polyacrylamide were used with 4% polyacrylamide stacking gels. Samples were suspended in sample buffer containing 2-mercaptoethanol and heated to 95°C for five minutes prior to running on polyacrylamide gels at 0.5 amps for 30-45 minutes. Proteins in the gels were transferred to nitrocellulose electrophoretically in a pH 8.5 Tris-glycine buffer. The nitrocellulose blots were

blocked overnight with PBS containing 2.5% bovine serum albumin (BSA) and 0.1% tween 20. After washing with PBS containing 0.1% BSA and 0.1% tween 20 (PBS-tween), the blots were exposed to rabbit IgG in PBS-tween for one hour at room temperature. The blots were washed, then exposed to 35ul ^{125}I-labeled protein A in PBS-tween for one hour. Unbound protein A was removed by washing, the blots were air dried, and autoradiographed for 2 - 10 days.

Quantitative dot-blot procedure

The quantitative dot blot was performed according to previously described methods (Glenney et al., 1982; Zanetti et al., 1991). PMNs were incubated with stimulants, then the PMNs were pelleted by centrifugation and 15ul of supernatant from each sample was blotted onto 1 cm nitrocellulose squares. Alternatively, proteins in the supernatant of stimulated PMNs or suspensions of lysed cells were precipitated with 10% TCA. Precipitated proteins were pelleted by centrifugation and resuspended in PBS or SDS-PAGE sample buffer. Fifteen microliters of resuspended protein from each sample was blotted onto 1 cm squares of nitrocellulose in triplicate. After blotting, the nitrocellulose squares were allowed to air dry. Proteins were fixed to the nitrocellulose by soaking for 30 minutes in 0.2 % glutaraldehyde in PBS, followed by a 15 minute quench in 50mM ammonium chloride in PBS. Unbound sites on the blots were then blocked by soaking overnight in PBS with 2.5% BSA and 0.1% tween. After blocking, the squares were rinsed in PBS and

exposed to rabbit IgG in PBS at room temperature for one hour. The squares were washed with PBS-tween, then soaked in 35ul of ^{125}I labeled protein A in PBS-tween for one hour. Unbound protein A was removed by washing with PBS-tween, and the radioactivity of each square was measured using a gamma counter.

Results

Seven rabbits were immunized with synthetic bac-KLH conjugate with adjuvant, and two were injected with synthetic peptide with adjuvant. Neither rabbit that received synthetic peptide developed antibodies against bactenecin. Two of the seven that received bac-KLH conjugate protein developed antibodies detectable by western blot that were not present before immunization. Figure 1 is a western blot depicting representative results of rabbit immunization with the bac-KLH conjugate and adjuvant. When lysed bovine PMNs were used as the antigen on western blots, newly formed antibodies bound to proteins of 7 and 20 kD molecular weight (figure 1a). When bac-KLH conjugate protein was used as the antigen on western blots, new antibodies in post-immunization sera bound to proteins on the nitrocellulose with one band at a molecular weight of less than 5 kD and multiple bands at greater than 50 kD (figure 1b). Neither rabbit's pre-immunization serum contained antibodies that bound to bac-KLH or lysed bovine PMN proteins of those molecular weights. Some rabbits developed post-immunization antibodies that bound lysed bovine PMN

proteins with molecular weights that did not correspond to bactenecins. One rabbit developed new antibodies that apparently recognized the synthetic peptide, but did not bind to native bactenecins from PMN lysates.

Serum from rabbits used in this study, as well as serum of rabbits from other sources, contained pre-existing antibodies that recognized several different proteins from lysed bovine PMNs (figure 1). It was expected that the anti-bactenecin antibodies could be separated from these naturally occurring antibodies. The affinity columns and protein A columns used to try to purify anti-bactenecin antibodies from sera did not effectively remove all of the naturally occurring antibodies. Adsorption to bovine PMNs or porcine PMN preparations also failed to remove all of the unwanted antibodies.

The most effective purification method used was an affinity column with bac-KLH conjugate coupled to Affigel-15. A Western blot of elution fractions from this column is shown in figure 2. The pH 3 elution fractions from this column contained antibodies that bound to lysed PMN proteins of 7 kD, 14.5 kD, and 20 kD. The antibodies forming these bands were assumed to be binding to Bac7, proBac7, and the 14.5 kD intermediary peptide. However, the same elution fractions also consistently contained antibody that bound a lysed bovine PMN protein of about 25 kD on Western blots. Antibody forming the 25 kD band was found in the pre-immune serum of this rabbit and in sera of all rabbits tested, regardless of their source. Antibody that formed this band could not be separated from anti-bactenecin antibody by any of the techniques attempted.

Figure 3 is a representative Western blot of supernatants of bovine PMNs exposed to various stimulants probed with unpurified immune rabbit serum. It appeared that supernatants from PMNs stimulated with PMA, OZ, or A23187 contained bactenecins. Supernatants from unstimulated cells typically showed no bands on western blots. Western blots of supernatants from PMNs stimulated with OZ or A23187 typically had a band at 7 kD. When neutrophils were stimulated with PMA, a band was usually seen at 20 kD. When blots of stimulated PMN supernatants were probed with whole serum from immunized rabbits, several bands were observed. Opsonized zymosan in particular seemed to cause exocytosis of numerous substances that bound naturally occurring antibody in rabbit serum. Figure 4 is a representative Western blot of supernatants of PMNs stimulated with PMA and probed with purified antibody. When purified antibody was used to probe the proteins from supernatants, usually only bands corresponding to bactenecin peptides were seen.

Discharge of bactenecins from stimulated neutrophils could not be consistently confirmed by quantitative dot blots. Gamma counts of dot blots of supernatants from stimulated cells often were higher than counts for unstimulated cells and background values, but the increase was generally small and was not consistent. In general, lysed PMNs had significantly higher counts than unstimulated cells or background counts, but this also exhibited large day-to-day variation.

Discussion

It appears that the synthetic bactenecin sequence coupled to KLH was immunogenic in some rabbits when injected with the adjuvant Titermax. Two rabbits out of seven given bac-KLH conjugate produced new antibodies to proteins of the appropriate molecular weight for bactenecins, while neither of two rabbits given only peptide with adjuvant produced detectable new antibodies. Immune sera reacted to proteins of less than 5 kD and produced multiple bands from about 50 to 60 kD on western blots when bac-KLH was the antigen. No other immunoglobulin binding was noted. The band at less than 5 kD is probably due to antibody binding to synthetic peptide that remained unconjugated to the KLH. The multiple bands at greater than 50 kD are probably due to antibodies formed to KLH. Hemocyanin of keyhole limpets is a protein of variable size, consisting of an indefinite number of subunits of approximately 50 kD. Each of these subunits should contain many possible sites capable of conjugating the smaller synthetic bactenecin (Maloy and Coligan, 1991). Each subunit of 50 kD may link a variable number of bac peptides, resulting in a large number of products of slightly different molecular weight.

Antibody from immune sera produced bands corresponding to the 7 kD mature Bac7 and the 20 kD proBac7 when lysed bovine PMNs were used as the antigen on a western blot. Occasionally a band that probably represents the 14.5 kD intermediate peptide was seen. Cleavage of the proBac7 to Bac7 occurs rapidly following cell lysis (Zanetti et al., 1990). Antibody binding to proteins of 7 kD,

20 kD, and 14.5 kD varied between PMN lysate preparations depending on the amount of time elapsed between cell lysis and protein precipitation by TCA. None of these bands were seen with pre-immune sera from the two rabbits.

Probactenecins must be cleaved by elastase to form the bactericidal Bac5 and Bac7. The neutrophil stimulant PMA induces exocytosis of large granules, but not primary granules (Scocchi et al., 1992). Under these conditions, bactenecins do not mix with elastase from the primary granule, cleavage of the proBac7 does not occur, and a band corresponding to the 20 kD proBac7 is the only band observed. This effect is seen in figure 4.

The enzymatic cleavage of probactenecins to mature Bac5 and Bac7 is affected by pH and by proteinase inhibitors. Lysing of bovine neutrophils allows the mixing of contents from the large and primary granules, exposing probactenecins to the enzymatic action of elastase. If cell lysis occurs at pH 7, the 20 kD proBac7 is converted to the 7 kD Bac7. Conversion is prevented or inhibited at pH 4 or in the presence of serine proteinase inhibitors (Zanetti et al., 1991). These effects were seen in this study (figure 5). A prominent band was seen at 20 kD and little or no reactivity at 7 kD when PMNs lysed at pH 4 were used as antigen on western blots and probed with rabbit IgG. The same pattern occurred when PMNs were lysed at pH 7 in the presence of the proteinase inhibitor PMSF. When PMNs were lysed at pH 7 in PBS, bands were usually seen at only 7 kD or at both 20 kD and 7 kD.

Western blots demonstrated that the IgG preparations were not pure. Blots of lysed cells always showed additional bands at molecular weights that could not be due to bactenecins. None of the methods used to purify antibody removed all non-specific antibodies. Bac5 and Bac7 have unusual amino acid compositions and unique sequences that make them unusual proteins (Frank et al., 1990). For this reason, it was expected that anti-bactenecin antibodies could be separated from other antibodies in the serum by affinity chromatography. In spite of the uniqueness of the bactenecins, it seems that the rabbits had naturally occurring antibodies that cross-reacted to epitopes present on synthetic bac-KLH conjugate. Pre-existing antibodies bound to the conjugated peptide preparation in affinity columns and could not be eluted from the columns independently of the anti-bactenecin antibodies. Several elution methods were used to attempt to separate antibodies that bound to the affinity columns, but none was completely successful. No pre-existing antibodies that bound to non-conjugated synthetic bactenecin were noted, but immune serum did contain antibodies that apparently recognized synthetic peptide. However, when the synthetic peptide was used in affinity columns, anti-bactenecin antibody did not bind to it to allow purification. Either synthetic peptide did not bind to the column matrix, or coupling to the matrix obscured the site that antibodies recognized.

We speculated that neutrophils of other species may contain structures homologous to bovine neutrophils that could bind the unwanted antibodies without binding anti-bactenecin immunoglobulins. To test this theory, immune rabbit serum was incubated with whole bovine PMNs as well as with intact and lysed porcine

PMNs. The adsorption technique removed some of the non-bactenecin antibodies, but not all.

Quantitative dot-blots probably were able to detect discharge of bactenecins. Western blotting indicated that rabbit IgG preparations contained anti-bactenecin antibody. Some antibody preparations were sufficiently pure that only proteins corresponding to bactenecins were observed in supernatants of stimulated PMNs (figure 4). However, the results of quantitative dot blots of supernatants had poor repeatability. The inconsistency of results may indicate that the methods used were not sensitive enough to measure the small amounts of bactenecin released by stimulated neutrophils.

Gamma counts of blots of lysed PMN preparations also had unacceptable day-to-day variability. Numerous combinations of lysing agents and techniques, blocking agents, and washing solutions were used, but none proved to have satisfactory repeatability. The impurity of the IgG may have contributed to the inconsistency. It is likely that the specificity of the IgG preparations was not sufficient to allow proper interpretation of any results that may have been obtained.

Sera from rabbits other than those used in this study and sera from other species were tested for the presence of naturally occurring anti-bovine neutrophil antibodies. All rabbit sera tested contained anti-bovine PMN antibodies. There was variation in number and location of the bands observed, but all rabbit sera tested contained antibodies that recognized a protein of approximately 25 kD. This may be

the same antibody that could not be separated from the antibactenecin antibody in this study.

It is possible that the 25 kD band may have been formed by an Fc receptor (FcR) or receptor subunit liberated from lysed PMNs. However, binding of IgG to its target antigen is necessary to cause structural changes in the Fc portion of the molecule that allow Fc receptor binding (Unkeless et al., 1992). For this reason, one might expect that unbound antibodies in solution would bind minimally to Fc receptors that may be present on the nitrocellulose blot. However, there may have been antibody complexes or aggregates formed in the preparations. Aggregation may cause the needed structural change to occur in enough IgG to bind to Fc receptors and form a band on the blot.

It is possible that rabbit IgG that is not bound to its antigen would bind to Fc receptors from lysed PMNs of other species. Adsorbing rabbit serum with lysed PMNs from pigs did not remove the offending molecules. The IgG fraction of rabbit serum consists almost entirely of IgG1, with very little IgG2 (Bankert and Mazzaferro, 1989). In cattle, it is the IgG2 subtype that binds selectively to neutrophil Fc receptors (Tizard, 1992). If rabbit IgG1 could bind to bovine FcR, protein A would have to be able to bind to a different site on the Fc portion of the molecule to form a band on Western blot. The exact binding locations of mammalian FcR and protein A are apparently unknown.

Three types of FcR have been described. No reports regarding the molecular weight of bovine FcR could be found. Mouse and human studies have reported that

FcRI, which is expressed at low levels in neutrophils, has a molecular weight of 70 kD. Fc receptor II and FcRIII are commonly found on neutrophils, and have molecular weights of 40 kD and 50-70 kD respectively (Tizard, 1992; Unkeless et al., 1992). None of the FcR described has the correct molecular weight to cause the persistent band noted on Western blots in this study. It is possible that a smaller subunit of FcR produced during PMN processing for Western blot could bind antibodies and produce the band.

Rabbit IgM can bind protein A at low levels (Bankert and Mazzaferro, 1989). Affinity chromatography using protein A to bind the IgG fraction may not eliminate IgM from the purified IgG preparation. If rabbit IgM were to bind protein A or to any proteins used in an affinity column, it would likely be eluted with IgG, bind proteins on the nitrocellulose, and may bind enough ^{125}I-labeled protein A to cause a visible band on the Western blot. This is another possible explanation for the persistent band at 25 kD.

Goat, guinea pig, and dog sera also contained antibodies that recognized lysed bovine PMN proteins of varying sizes. None of the sera tested contained immunoglobulins that bound to proteins of 7 kD or 20 kD. Sera from mouse and turkey showed no antibody reactivity to lysed bovine PMNs. Perhaps a species other than rabbits could be used to produce anti-bactenecin antibodies with fewer problems of purification, or a mouse monoclonal anti-bactenecin could be developed. A monoclonal antibody that recognizes only Bac5, Bac7, and their precursors has been reported (Marzari et al., 1988). Perhaps this monoclonal antibody could be obtained

for further studies. However, the authors of that paper did not report using this monoclonal in subsequent research.

It is possible that synthetic peptide was not the best choice as an immunogen for this purpose. Since only a portion of the molecule was synthesized, its tertiary structure may have been different from the complete peptide. Others have purified the Bac5 and Bac7 peptides from granule fractions of bovine neutrophils and used these native proteins to immunize rabbits. Mono-specific anti-Bac7 antibody was purified from serum by affinity chromatography using native Bac7 linked to Affigel 10 (Zanetti et al., 1990). They reported no problem separating the antibactenecin immunoglobulin from other antibodies in the IgG fraction of the serum. The method of isolating bactenecins from granule extracts is laborious (Gennaro et al., 1983b), but may result in a more suitable immunogen than the synthetic peptide.

The quantitative dot-blot technique has been used successfully in research, but it has some drawbacks to its use. It employs radioactive materials, it is labor intensive and time consuming, and it is not easily adapted to test large numbers of samples. If quantities of antibody of sufficient purity and specificity can be obtained, perhaps an ELISA for determination of bactenecin can be developed to address these concerns.

Due to the inability to develop a sensitive, quantitative assay, this study was unable to provide any additional information concerning the regulation of the bovine neutrophil third granule. It appears that the antibodies produced were pure enough to measure the amount of bactenecin in the supernatant of stimulated neutrophils.

However, the failure to produce mono-specific antibody preparations prevented an accurate determination of the total bactenecin content of lysed neutrophil samples, which made calculation of the percent discharge of bactenecin impossible. This is an interesting subject worthy of further research. The large granule is unique to ruminants, and its regulation is poorly studied. Large granules and their antimicrobial proteins are believed to be critical to the health of domestic cattle (Gennaro et al., 1989). Inhibition of degranulation of the large granule may predispose cattle to bacterial infection. Knowledge of the regulation of granule discharge may enable the development of therapeutic drugs to aid in preventing or treating bacterial diseases in cattle.

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Figure 1a

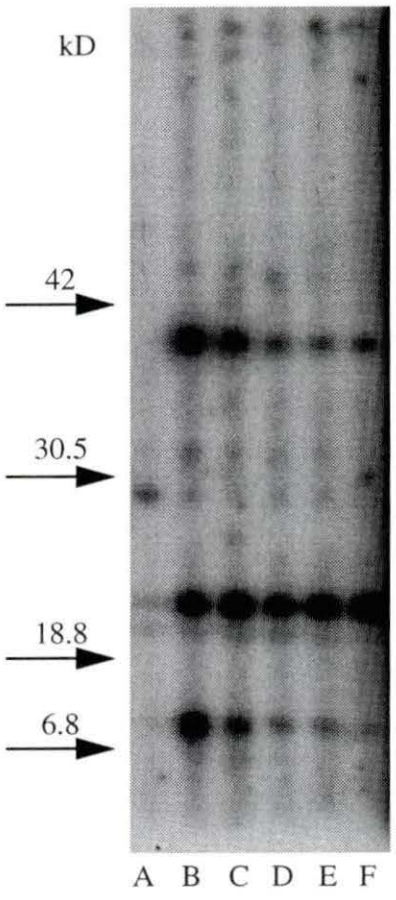

Figure 1b

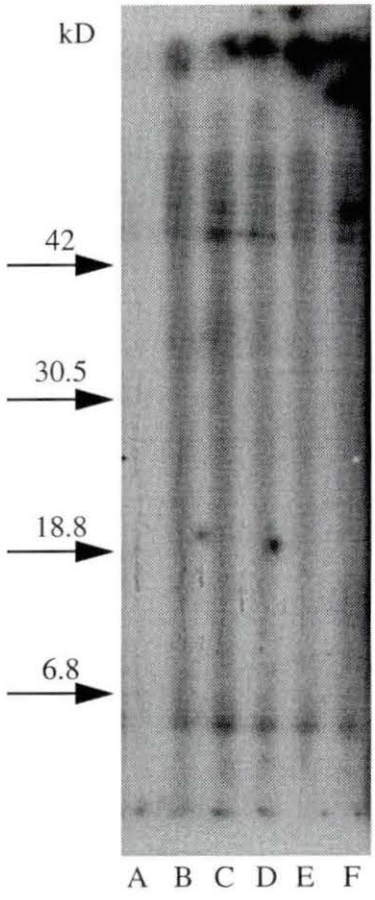

Figure1. Western blot analysis of the development of antibodies in a rabbit following immunization with synthetic bactenecin conjugated to KLH, injected with Titermax adjuvant. Figure 1a shows antibody reactivity to lysates of bovine neutrophils. Figure 1b shows antibody reactivity to the bac-KLH conjugate protein preparation. Cell lysates and bac-KLH preparations were precipitated in TCA and prepared for SDS-PAGE and Western blot. In both figures, column A is pre-immunization serum; B is 3 weeks post-immunization (PI); C is 6 weeks PI; D is 9 weeks PI; E is 12 weeks PI; F is 15 weeks PI.

Figure 2

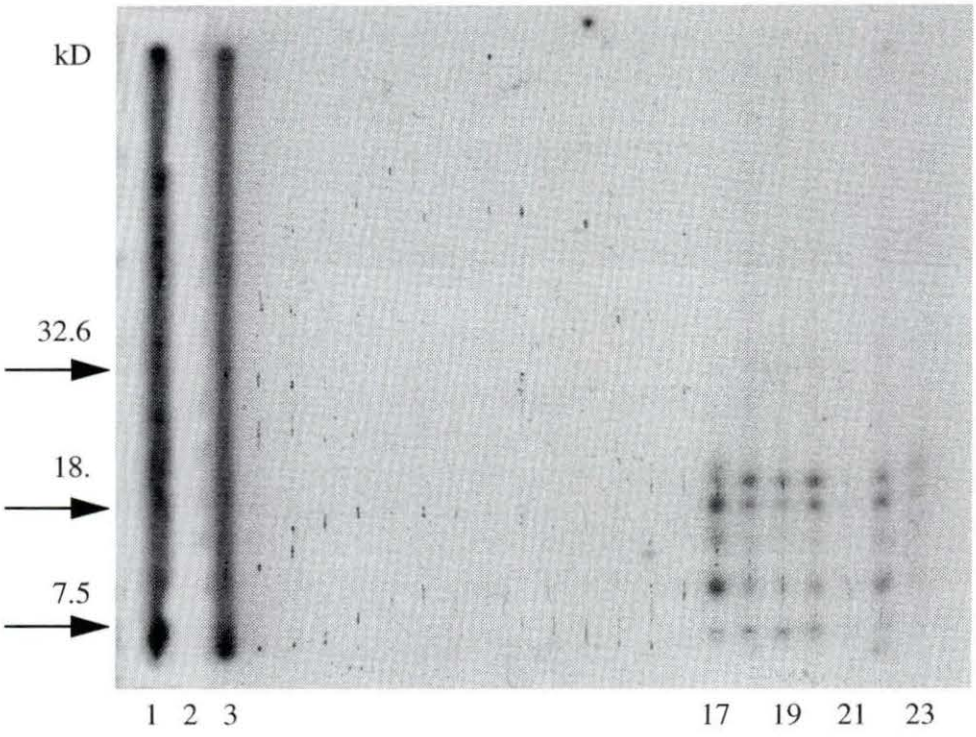

Figure 2. Western blot analysis of an attempt to isolate mono-specific anti-bactenecin antibody from an affinity column. The column matrix was bac-KLH bound to Affigel-15. Proteins from bovine PMN lysates were precipitated with TCA and processed for SDS-PAGE and Western blot. Heat-inactivated immune rabbit serum, shown in column 1, was incubated in the column for two hours. Column 2 was left empty. Columns 3 through 17 are representative fractions eluted with ten column volumes of isotonic HEPES buffer at pH 7. Columns 17 through 23 are sequential fractions eluted with citrate buffer at pH 3.

Figure 3

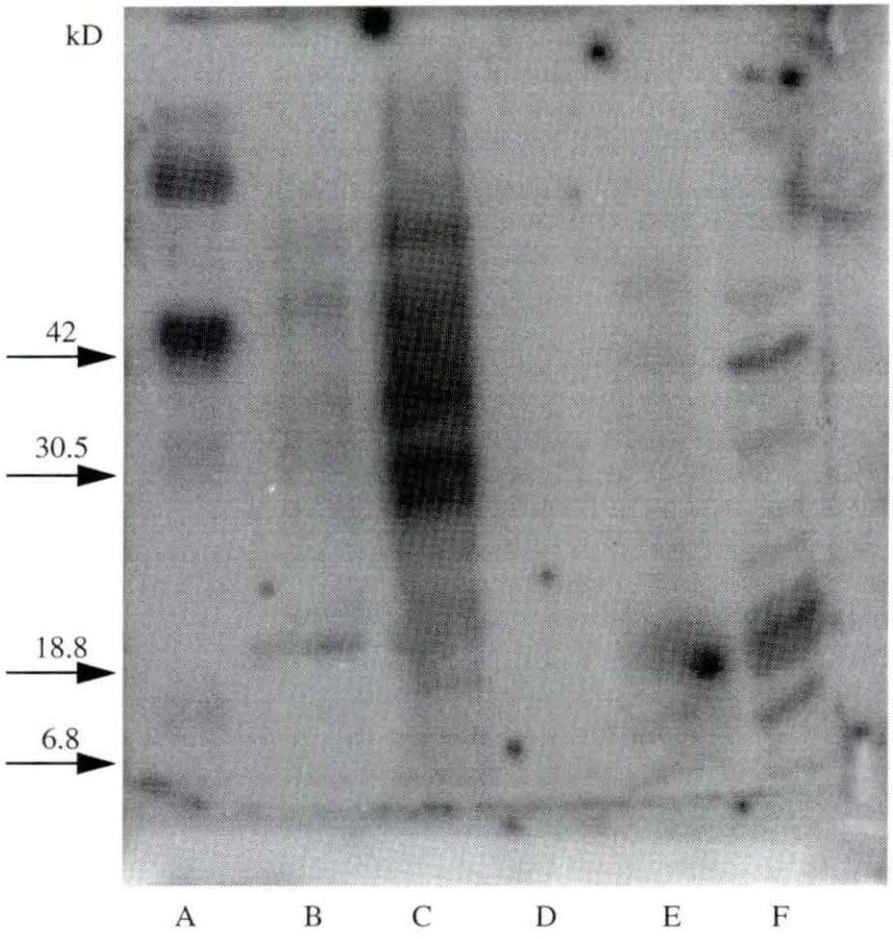

Figure 3. Western blot analysis of supernatants of stimuated, unstimulated and lysed bovine PMNs. Cell-free supernatants or cell lysates were TCA precipitated, and processed for SDS-PAGE and Western blot. The blot was exposed to whole post-immunization rabbit serum. A, molecular weight markers; B, supernatant of PMNs stimulated with 100nM PMA; C, supernatant of PMNs stimulated with opsonized zymosan; D, supernatant of unstimulated PMNs; E, supernatant of PMNs stimulated with calcium ionophore; F, PMN lysate.

Figure 4

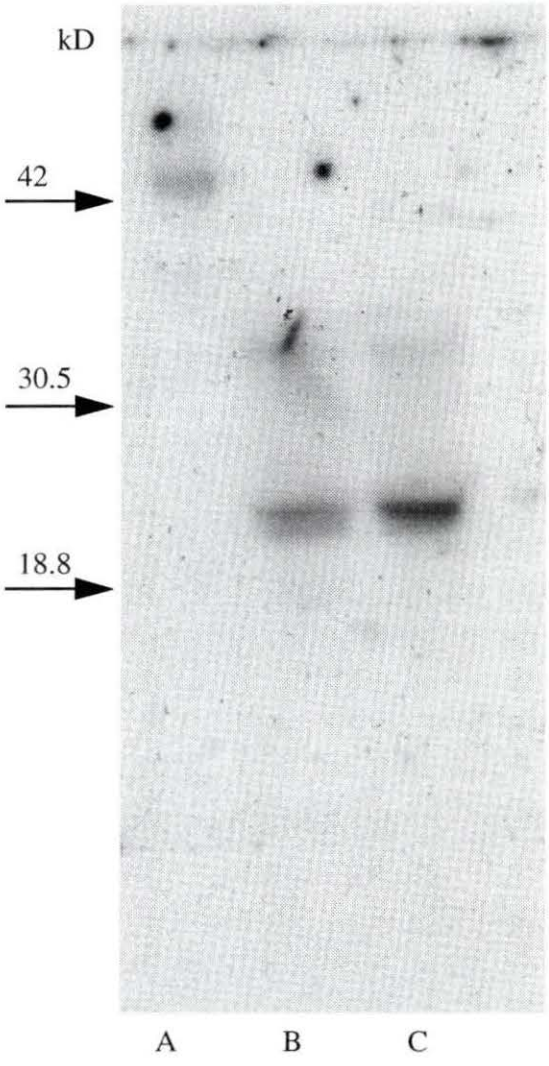

Figure 4. Western blot analysis of supernatants of bovine PMNs stimulated with PMA. Proteins in the supernatants were TCA precipitated and prepared for SDS-PAGE and Western blot. The blot was exposed to affinity purified rabbit serum. Column A, molecular wight markers; B, supernatant of PMNs stimulated with 50nM PMA; C, supernatant of PMNs stimulated with 100nM PMA.

Figure 5

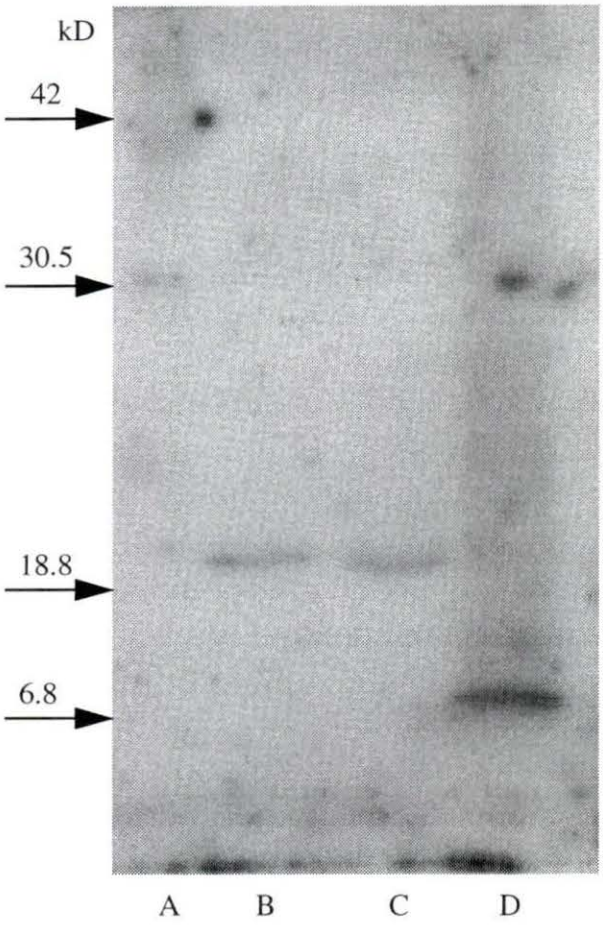

Figure 5. Western blot analysis of bovine PMNs lysed at acid pH, or neutral pH with or without proteinase inhibitor. Cell lysates were precipitated with TCA and prepared for SDS-PAGE and Western blot. The blot was exposed to affinity purified rabbit serum. Column A, molecular weight markers; B, PMNs lysed at pH 3; C, PMNs lysed at pH7 in PBS in the presence of 1mM PMSF; D, PMNs lysed at pH7 in PBS.

GENERAL CONCLUSIONS

Degranulation is a vital antimicrobial function of neutrophils. Inhibition of degranulation may contribute to increased susceptibility to bacterial infection. Preventing or reversing inhibition of degranulation may be beneficial in fighting bacterial infection. Neutrophil granule contents may cause damage to healthy tissue. Preventing inappropriate degranulation may reduce tissue injury in some cases. A comprehensive understanding of the regulation of granule discharge may enable the development of therapy to enhance or inhibit degranulation as needed. The results of the research described here may enhance the knowledge of the regulation of granule release.

The assay to measure myeloperoxidase release should enable investigators to measure primary granule discharge of a large number of samples directly, quickly, and quantitatively, without using radioactive or highly toxic materials. This assay may facilitate large scale studies of the regulation of primary granule degranulation.

It has been reported that *Brucella abortus* and *Haemophilus somnus* inhibit the degranulation of bovine neutrophil primary granules. This mechanism may be important to the pathogenesis of the diseases caused by these pathogens. The myeloperoxidase assay was used to obtain evidence to support this proposed virulence mechanism.

It has been reported that a possible reason that the neutrophils of calves have reduced antibacterial abilities compared to adult cattle may be due to a reduced

myeloperoxidase content. However, there are conflicting reports regarding the total myeloperoxidase content of calf neutrophils relative to adult neutrophils. The myeloperoxidase assay described here was used to provide additional data indicating that calf neutrophils contain less myeloperoxidase than adult neutrophils. The assay also provided some data not reported previously. It was observed that although calf neutrophils contain less total myeloperoxidase, they appear to respond to opsonized particles more vigorously than adult neutrophils, discharging a larger proportion of the enzyme at a more rapid rate. It is unknown if this difference is of physiological significance. It is also unknown if this difference is due to increased numbers of surface receptors, increased sensitivity of a component of the signal transduction mechanism, or some other factor. Future research could attempt to determine the significance and the reason for this apparent difference in regulation.

Corticosteroids, BVD virus infection, and several other agents inhibit the iodination reaction of bovine neutrophils. Iodination is an indirect measure of primary granule degranulation. The myeloperoxidase assay directly measures primary granule discharge, and could be used to determine if these inhibitory factors reduce degranulation or if they interfere with some other aspect of the chain of events necessary for iodination. Such information could more accurately define the mechanisms of action of these agents.

The large granule is found only in the neutrophils of ruminants. This granule type and its unique antimicrobial peptides, the bactenecins, may be important in antibacterial defense in domestic cattle, yet the regulation of its degranulation is

poorly studied. This research intended to expand the knowledge of stimulants, inhibitors, and enhancers of large granule degranulation. Information of this type should aid in describing the intracellular signal transduction pathways involved in the regulation of large granule discharge. Technical problems in validating the assay prevented the gathering of any meaningful data.

The regulation of degranulation of bovine neutrophils warrants further research. Sufficient knowledge of how granule discharge is controlled could lead to therapeutic agents to enhance or inhibit degranulation as needed. Such drugs could be useful to combat bacterial infections or to prevent inappropriate tissue injury by neutrophils. The research reported in this thesis may have contributed to such knowledge. The myeloperoxidase assay described in the first manuscript has several advantages over other assays described to measure primary granule degranulation. The assay could be a valuable tool for gathering additional information concerning the regulation of the primary granule. It was used to obtain data not previously reported; the primary granules of calf neutrophils discharge faster and more completely than neutrophils from adult cattle, perhaps indicating a difference in regulation. The research regarding the regulation of the large granule of the bovine neutrophil was intended to add considerable knowledge to a poorly studied subject. Unfortunately, no meaningful data were obtained due to technical difficulties.

ACKNOWLEDGEMENTS

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