THE INFLUENCE OF *PASTEURELLA HAEMOLYTICA* AND ITS PRODUCTS ON BOVINE NEUTROPHIL FUNCTION

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ABSTRACT

THE INFLUENCE OF *PASTEURELLA HAEMOLYTICA* AND ITS PRODUCTS ON BOVINE NEUTROPHIL FUNCTION

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Pneumonic pasteurellosis is a fulminant respiratory disease of ruminants caused by *Pasteurella haemolytica* (PH) serotype A1. Neutrophil depletion prevents pulmonary injury by *Pasteurella haemolytica* affirming that products produced or released by neutrophils are important factors in the pathogenesis of pneumonic pasteurellosis. To investigate the effects of *Pasteurella haemolytica* and its products on bovine neutrophil function, neutrophils were isolated from neonatal calves to define and compare: 1) oxidative responses of bovine neutrophils to selected agonists and to *Pasteurella haemolytica*, 2) effects of *Pasteurella haemolytica* and *Escherichia coli* endotoxins on the oxidative responses of bovine neutrophils, and 3) release of cytoplasmic granules and enzymes by bovine neutrophils in response to agonists and to *Pasteurella haemolytica*.

Luminol-enhanced chemiluminescence and superoxide production were measured to quantitate bovine neutrophil oxidative metabolism. Latex particles, phorbol ester,

calcium ionophore, and opsonized zymosan were used as agonists.

Opsonized zymosan and opsonized live *Pasteurella haemolytica* enhanced chemiluminescence and superoxide production by neutrophils, but these responses were exceeded by those exposed to latex, phorbol ester and calcium ionophore.

Exposure of neutrophils to *Pasteurella haemolytica* or *Escherichia coli* endotoxin enhanced superoxide production in response to phorbol ester, calcium ionophore, and opsonized zymosan, but chemiluminescence was diminished when the agonists were phorbol ester or calcium ionophore.

The greatest release of beta-glucuronidase, vitamin B_{12}-binding protein and cytosolic lactate dehydrogenase occurred in neutrophils exposed to live-opsonized *Pasteurella haemolytica*.

These results indicate that: 1) live *Pasteurella* organisms and opsonins facilitate the release of reactive oxygen species and superoxide anion from neutrophils, 2) the production of superoxide, by neutrophils, is enhanced by exposure to trace quantities of endotoxin, and 3) live organisms and opsonins markedly enhance the release of B12BP (specific granules) partially through the lysis of neutrophils. It is postulated that these events may have major roles in the neutrophil-mediated injury in pneumonic pasteurellosis.

To Alga "Shorty" and Marjorie Watson,

my parents, and Ellie, my wife

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INTRODUCTION

Pneumonic pasteurellosis caused by the gram negative bacterium *Pasteurella haemolytica*, has a primary role in the bovine respiratory disease complex. Slocombe et. al.[129] recently determined that this disease is neutrophil-mediated. Neutrophil-mediated injury has been attributed to the production of reactive oxygen species, including superoxide anion and its metabolites, the release of enzymes from cytoplasmic stores, and the production of metabolites of arachidonic acid (prostaglandins and leukotrienes). Due to the complexity and interrelationships of diverse pathways active *in vivo*, it was felt necessary to investigate the interactions of *Pasteurella* and neutrophils by *in vitro* methods.

In my experiments I decided to investigate the production of oxidative metabolites and enzyme release, by neutrophils, in response to the *Pasteurella* organism. Studies of the oxidative metabolism of bovine neutrophils were designed to investigate the capacity of stimulated bovine neutrophils to produce superoxide anion and other reactive oxygen metabolites. To characterize the magnitude of bovine neutrophil responses it was necessary to define a

battery of agonists which would provide consistent and maximal responses in bovine neutrophils. It was hoped that this series of agonists would also provide a "frame of reference" for planned comparisons of the responses to the *Pasteurella* organism.

A recent publication by Guthrie et. al.[77] described a phenomenon induced by the exposure of neutrophils to trace quantities of bacterial lipopolysaccharide (endotoxin), termed "priming", where exposed cells markedly increased the production of superoxide in response to a diverse series of agonists. Since the *Pasteurella* organism has the capability of producing endotoxin, a series of experiments were performed to investigate whether *Pasteurella* endotoxin might induce similar responses in bovine neutrophils. These experiments utilized the agonists defined in the oxidative metabolism protocols as stimuli.

Lastly, the same agonists used in the investigations of oxidative metabolism and the effects of endotoxin were used to determine the magnitude of enzyme release by bovine neutrophils. Subsequently, the responses to these agonists were compared to those initiated by exposure of neutrophils to the *Pasteurella* organism.

It was hoped that these *in vitro* experiments would provide information about the role of neutrophil products in the pathogenesis of pneumonic pasteurellosis, and serve as a basis for future investigations into means and methods for modulating or preventing the disease.

LITERATURE REVIEW: PART I: MECHANISMS OF NEUTROPHIL ACTIVATION (TRANSDUCTION)

INTRODUCTION: Within the past five years more than 25,000 publications have emphasized various aspects of neutrophil function. The majority of these publications centered upon the cellular biology of the neutrophil and attempted to define the mechanisms of neutrophil-mediated injury. Few of the studies were related to the general features of the bovine neutrophil or to the specifics of neutrophil-mediated diseases in the bovine species. This literature review will be divided into two portions: a summary of the currently understood mechanisms of neutrophil transduction or activation and a review of the current literature which specifically applies to the bovine neutrophil. A major assumption is that while the bovine neutrophil may possess phenotypic differences in enzymic components, and perhaps in biological behavior, the biochemical nature of the transduction mechanisms, although not in actuality defined, are essentially similar. While the principal thrust is in the exploration of (bovine) neutrophil oxidative metabolism and enzyme release, due to the complexity of interrelated pathways, a review of those

aspects of neutrophil function cannot be separated distinctly from other functions initiated simultaneously.

MECHANISMS OF NEUTROPHIL TRANSDUCTION: There are many excellent reviews of neutrophil function[1-4] and the role of the neutrophil in inflammation[5,6], all providing a basis from which to initiate a review of the recently defined aspects of neutrophil activation or transduction.

NEUTROPHIL RESPONSE PHASES: The neutrophil responses to external stimuli occur in three phases.[6] Phase I occurs through ligand binding resulting in altered membrane topography with exposure of surface sialic acid residues, glycoproteins and receptors. Phase II involves the opening of ion channels or molecular pores with exposure of ion pumps, transport processes, or surface enzymes. Altered ionic permeability and cation fluxes may trigger phase III directly or indirectly through the formation of second messengers. The phase III response leads to activation of cryptic enzyme systems which control or mediate membrane fusion. Phase II ligands preferentially activate the energy producing pathways including glucose transport, glycolysis and glycogenolysis whereas phase III ligands activate energywasting or catabolic pathways.

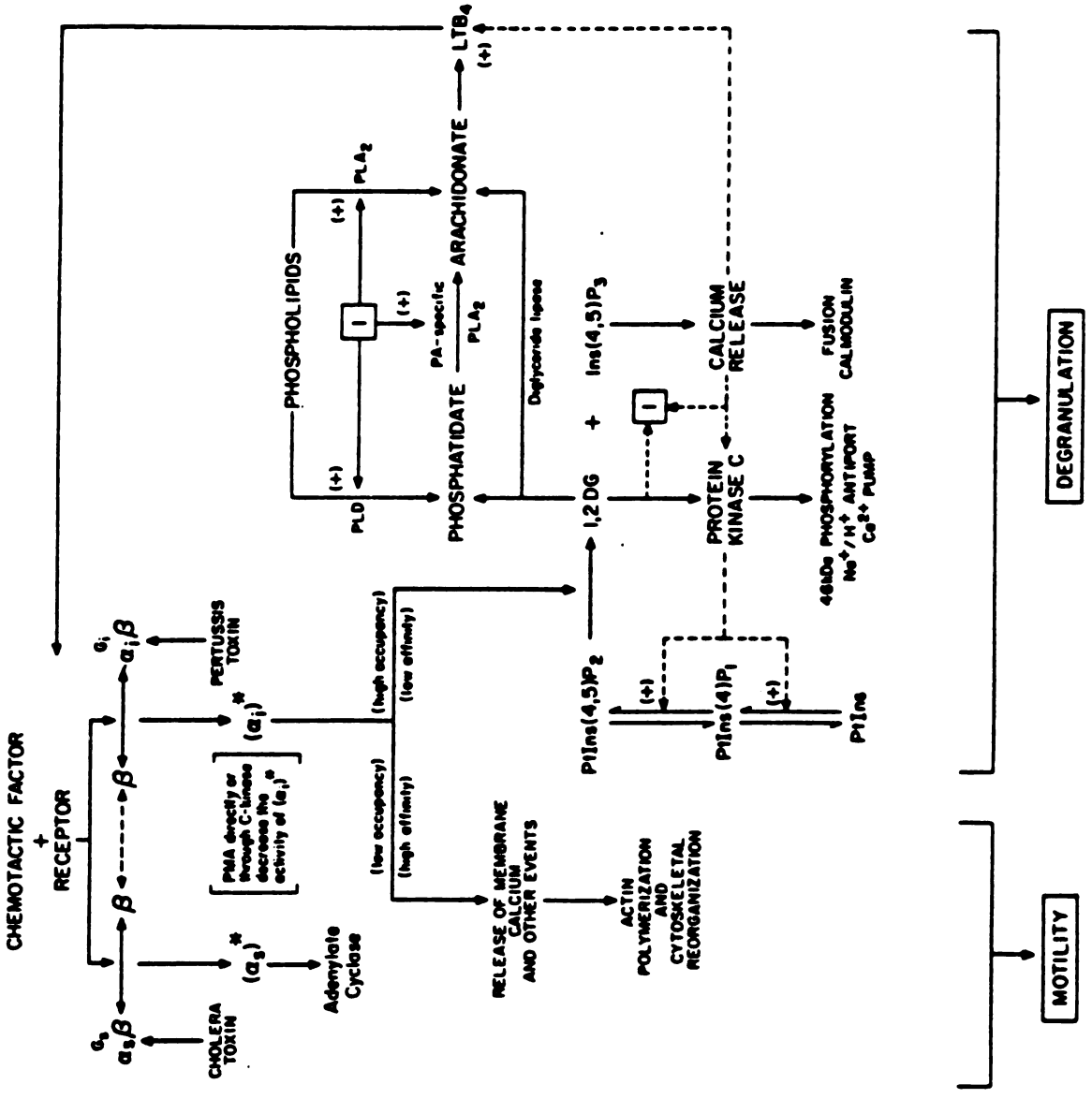
INTRACELLULAR SECOND MESSENGERS: Central to the activation of neutrophils are the intracellular second messengers, the divalent cation Ca^{2+} and products of polyphosphoinositide metabolism.[4,7-9] The complexity of this

activation is exemplified by the fact that phospholipase C, the enzyme necessary for formation of the two primary second messengers diacylglycerol (DAG) and inositol triphosphate (IP_3), is itself a Ca^{2+}-dependent enzyme. DAG in combination with Ca^{2+} and a phosphatidylserine (PS) substrate are the endogenous activators of the intracytoplasmic enzyme protein kinase C (PKC).[7] IP_3 is important in the release of intracellular calcium stores from the endoplasmic reticulum.[9] Recently studies have defined the presence of a 3-kinase, activated by a calmodulin-/Ca^{2+}-complex, which converts IP_3 to inositol tetrakisphosphate (IP_4) which appears to modulate the entry of calcium from the outside of the cell, through effects on calcium pumps. Also identified was a 5-phosphomonoesterase, activated by protein kinase C, which catalyzes the formation of IP_2, thereby terminating the second messenger response.[10] Divalent calcium has a major role as a cofactor for intracytoplasmic enzymes including phopholipase C and protein kinase C, as mentioned, but also for other enzymes important in stimulus-mediated events, including phospholipase A_2, calmodulin, and Ca^{2+}-requiring thiol proteinases (calpains). Calcium is also required for microtubule assembly and for actin and myosin interaction, necessary for leukocyte motility. This triumvirate also has a role in the migration of lysosomal granules to form phagolysosomes and for the migration of secretory granules to the cell surface.[6]

RECEPTOR BASED ACTIVATION: The response of neutrophils to chemotactic factors [formyloligopeptides (FMLP)], anaphylatoxins (C3b, C5a), leukotrienes, and platelet-activating-factor (PAF), are mediated through GTP-binding regulatory proteins, effected through the activation of phospholipase C (PLC).[11] A current perspective of receptor-mediated events is included as figure 1.[11] An intracellular pool of cell surface receptors is currently felt to reside within the membranes of specific granules[12-15], where the process of degranulation results in the addition of new plasma membrane and is associated with the mobilization of surface receptors.[14] Receptor stimulation leads to the hydrolysis of membrane polyphosphoinositides into DAG and IP_3. Pertussis toxin (PT) has been used as a probe of guanyl regulatory proteins since it ribosylates and blocks the inhibitory arm (N_i) of the guanyl proteins. This blockage acts at steps responsible for receptor-induced calcium mobilization and for the activation of protein kinase C.[11] However, PT does not affect the increase of free intracellular Ca^{2+} induced by platelet activating factor (PAF), but completely inhibits enzyme secretion.[16] PT almost completely abolishes the breakdown of membrane phosphoinositides, suggesting that N_i is intimately coupled to the activation of PLC. PT also prevents the release of arachidonic acid caused by chemotactic factors, which may be due to: a) lack of activation of

Figure 1. Overview of the Mechanisms of Receptor-Mediated Activation of Neutrophils: Diagrammatic Representation of the Early Events of Neutrophil Activation by Chemotactic Factors and the Postulated Points at Which Pertussis Toxin Acts.

PLA_2 and PLD are abbreviations for phospholipases A and D respectively. PtIns, PtIns4(P), and $Ins(4,5)P_3$ represent phosphatidylinositol and its mono- and trisphosphate forms respectively. From ref 15.


CHEMOTACTIC FACTOR
+
RECEPTOR
CHOLERA TOXIN
PERTUSSIS TOXIN
Adenylate Cyclase
PMA directly or through C-kinase decrease the activity of $(\alpha_i)^*$
(high occupancy)
(low affinity)
(low occupancy)
(high affinity)
RELEASE OF MEMBRANE CALCIUM AND OTHER EVENTS
ACTIN POLYMERIZATION AND CYTOSKELETAL REORGANIZATION
PHOSPHOLIPIDS
PLD
PLA_2
PA-specific PLA_2
PHOSPHATIDATE
ARACHIDONATE
LTB_4
Diglyceride lipase
1,2 DG
$Ins(4,5)P_3$
$PIIns(4,5)P_2$
$PIIns(4)P_1$
PIIns
PROTEIN KINASE C
CALCIUM RELEASE
FUSION CALMODULIN
48kDa PHOSPHORYLATION
Na^+/H^+ ANTIPORT
Ca^{2+} PUMP
DEGRANULATION
MOTILITY

phospholipase A_2 (PLA_2), b) decreased Ca^{2+}, or c) indirectly, due to decreased formation of DAG related to PLC alterations with less available substrate. In general, most surface receptor-mediated events are accompanied by increased levels of intracellular calcium.[17] This series of intracellular events keys the induction of oxidative metabolism, as well as motility and degranulation, and is felt by most investigators to be mediated by the activation of protein kinase C.

PROTEIN KINASE C: Several excellent reviews of protein kinase C (PKC) and its role in cell and neutrophil function have recently been published.[18-20] This ubiquitous intracytoplasmic enzyme is itself a receptor and is calcium and phospholipid-dependent. The enzyme is activated exogenously by phorbol esters (PMA) and endogenously by DAG, Ca^{2+}, and phospholipids (preferentially phosphatidylserine).[18-22] Experiments which used a series of synthetic diacyglycerols demonstrated a marked degree of variability in the induction of motility, superoxide production and enzyme release in treated cells[23], detailing the complexity and spectrum of PKC-induced cell responses. The activation of PKC is thought to be biochemically dependent on Ca^{2+}, but under some conditions is physiologically independent (see below).[19] Maximal activation of PKC has been induced experimentally by the concomitant use of PMA and calcium ionophore (CI, A23187), and has been postulated

to duplicate "normal" intracellular events.[24] In contrast to the chemotactic agents (summarized above) the use of pertussis toxin has no effect on PKC-mediated events, suggesting that the activation of this enzyme is distal to its effect.[22] In response to micromolar concentrations of calcium, the cytosolic form of PKC and a Ca^{2+} requiring thiol proteinase (calpain) become associated with the neutrophil membrane. Calpain activation converts PKC into a proteolytically modified Ca^{2+}-phospholipid-independent form which is released into the cytosol, where it has access to other, but presently undefined, protein substrates.[25-27] Some data suggest that PKC may be activated by two separate systems with differing substrate targets. Inhibitors of phophoinositide turnover inhibit chemotaxis and phagocytosis and may interfere in the activation sequence of the respiratory burst but there is no direct evidence linking phospholipid turnover to activation of the burst by PMA.[28,29] A major function of PKC appears to be related to the feedback control of cell surface receptors, termed down regulation. In neutrophils, feedback control over receptors coupled to inositol phospholipid breakdown has been suggested.[20]

THE RESPIRATORY BURST: Neutrophils, responding to soluble and particulate stimuli undergo a process termed the "respiratory burst" consisting of a rapid increase in oxygen consumption, glucose metabolism, and increased

hexose monophosphate (HMP) shunt activity. This activation coincides with induction of the respiratory burst enzyme, NADPH-oxidase[30,31], which is of major importance in cell killing and central to the production of reactive oxygen species. The mechanisms of formation and activation of this enzyme complex have only been partially defined through intense research. The current postulate is that a segment of the enzyme complex migrates with the membranes of specific granules, incorporating a b-cytochrome component into the membrane-bound enzyme complex thereby causing activation in conjunction with cytosolic calcium.[32] The activation of PKC has been postulated to be the principal event in initiation of NADPH-oxidase function, although an as yet unidentified cytosolic factor also appears to be required. The initial characterization of the factor(s) has shown that although it is Mg^{2+}-dependent, phospholipid, ATP, and GTP were not involved. The activation of the oxidase by arachidonic acid (AA), oleic acid, and sodium dodecyl sulfate (SDS) also requires this factor, and has been shown to be independent of PKC.[33] Cessation of enzyme activity is modulated, in part, through products of the myeloperoxidase-halide system.[34,35]

The electrons utilized to produce reactive oxygen species arise from the activation of the hexose monophosphate (HMP) shunt which not only supplies the reducing agent (NADPH) but generates one proton for each electron required

to reduce molecular oxygen to superoxide.[36] Superoxide is then metabolized into hydrogen peroxide spontaneously or through the activity of cytoplasmic superoxide dismutase (SOD).[1,5] Numerous enzyme systems have the ability to generate superoxide anion, which may act as a reductant or a oxidant depending upon its substrate.[1] Other reactive oxygen moieties which may be formed by activated phagocytic cells include hydroxyl radical ($OH\cdot$)[37,38], singlet oxygen ($^1O_2$)[39-42] and hypochlorous acid (HOCL)[43,44]. The proposed mechanism of $OH\cdot$ formation is termed the Fenton reaction, in which superoxide interacts with an oxidized trace metal (iron), causing reduction of the metal and O_2 generation. Subsequent reaction of the reducing form of the metal with hydrogen peroxide forms the hydroxyl anion ($OH\cdot$) and the hydroxyl radical (OH^-).[45,46] Lactoferrin, a component of cytoplasmic granules, may also supply iron as a catalyst to this reaction.[47] Hydroxyl radical may also be formed by the interaction of lipid peroxides with superoxide.[48] Singlet oxygen has been postulated to be generated by the interaction of hypochlorite, which is formed by the reaction of myeloperoxidase and H_2O_2 in the presence of halides, and hydrogen peroxide.[49] The amounts of superoxide and hydrogen peroxide formed are species- and stimulus-specific and vary with experimental conditions.[48]

Particulate agonists, such as opsonized zymosan (OZ), may activate the phosphoinositide (PI) cascade but stimulate superoxide release by a mechanism distinct from PMA, in that the stimulus is PLA_2-mediated, calcium-dependent and independent of PKC.[50] Arachidonic acid (AA) release is Ca^{2+}-mediated in OZ-stimulated cells but not in PMA treated.[51] Inhibitors of AA metabolism (BW755C and indomethacin) diminish AA release and superoxide production in OZ-stimulated cells. Interestingly, exogenous AA stimulates the respiratory burst by the same PLA_2-dependent pathway, but the inhibition of AA metabolism has no effect on superoxide production.[52]

QUANTITATION OF RESPIRATORY BURST ACTIVITY: Studies of the respiratory burst have consisted of the quantitation of oxygen consumption, production of superoxide anion, production of hydrogen peroxide, and activation of the hexose monophosphate shunt.[1,5,53,54] Other measurements have included: 1) the emission of incident light (chemiluminescence), enhanced by the use of amplifiers such as luminol or lucigenin[55-60]; 2) iodination, which is dependent upon phagocytosis, the respiratory burst, and myeloperoxidase leading to the formation of hypohalous acids and 3) nitroblue tetrazolium (NBT) reduction which quantitates the formation of an insoluble formazan from the reaction of the dye with superoxide, microscopically or spectrophometrically.[1,5,48,53,54]

The formation of superoxide which involves numerous enzyme systems and other mechanisms has been documented (Table 1).[1] Superoxide, which exists primarily as O_2- at neutral pH, is formed either by the univalent reduction of oxygen or by the univalent oxidation of hydrogen peroxide.[1] Superoxide can either lose or gain an electron, and therefore act either as a reductant or oxidant, forming either oxygen or hydrogen peroxide respectively.[1] The production of superoxide is quantitated by the superoxide dismutase-inhibitable reduction of ferricytochrome c.[1,5,53,54]

The generation of chemiluminescence (CL) by phagocytic cells has been attributed to the production of compounds such as superoxide anion (O_2-), singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2), hypochlorous ion ($HOCL^-$) and hydroxyl radical ($OH\cdot$).[1,55-57] The use of compounds such as luminol, a cyclic hydrazide which is oxidized to an electronically excited intermediate state (aminopthalate ion) and emits light upon relaxation to a ground state, has been used to augment "native" chemiluminescence and increase sensitivity.[58-60] Generated CL has been utilized in studies of cellular oxidative metabolism and in the delineation of metabolic or acquired opsonophagocytic dysfunction.[60]

MECHANISMS OF TISSUE DAMAGE AND CELL PROTECTION: The precise mechanisms by which oxygen-derived free radicals

Table 1. Mechanisms of Formation of Superoxide Anion

Xanthine oxidase	Oxidation of tetrahydropterines
Aldehyde oxidase	Oxidation of epinephrine at alkaline pH
Dihydroorotic dehydrogenase	Oxidation of ferredoxins
L-amino acid oxidase	Oxidation of cytochrome P450
NADPH cytochrome C dehydrogenase	Oxidation of hemoglobin or its isolated alpha or beta chains
Aerobic oxidation of dihydroxyfumaric acid and NADPH by peroxidase	Electrolysis
Lipoyl dehydrogenase	Photolysis
Glutathione reductase	Pulse radiolysis
Oxidation of reduced flavins	Ultrasonication
Oxidation of quinones	Reversal of superoxide dismutase reaction
Oxidation of thiols	Reaction of hydrogen peroxide with ceric ions
Oxidation of phenazine methosulfate	Spontaneous decomposition of hydrogen peroxide
Oxidation of streptonigrin	
Oxidation of phenothiazine derivatives	
Oxidation of dialuric acid	
Oxidation of 6-hydroxydopamine	
Oxidation of ferricytochrome C	

[a]Table modified from page 290, Klebanoff and Clark, Reference 1*;

induce cell injuries are unclear, but most experimental evidence suggests that lipid peroxidation of the cell membrane has an important role. Compounds active in serum which have major roles in the protection against oxidants include the copper-containing protein ceruloplasmin[61-63] and the iron-free fraction of transferrin.[64] Membrane-bound vitamin E has the ability to terminate free radical reactions by competing for peroxy free radicals (ROO·), and is found in highest concentrations in membranes associated with oxidative processes (mitochondria).[65,66] Cytosolic superoxide dismutase[67] has a principal protective role against the superoxide anion, preventing injury induced by this compound. The catabolism of hydrogen peroxide involves the enzymes catalase and glutathione peroxidase. It appears that catalase is only active when hydrogen peroxide levels are extremely high. Otherwise it assumes its normal function as a peroxidase with suitable electron donors.[68] The principal cytosolic protection is derived from glutathione peroxidase[68,69], an enzyme which exists in selenium-dependent and selenium-independent forms.[70] The latter appears only to be important in the detoxification of organic radicals, not hydrogen peroxide. The distribution of the enzyme subtypes differs widely within species.[70] The activity of this enzyme is closely tied to the HMP shunt and the availability of NADPH and glutathione reductase. Therefore, the HMP shunt has a regulatory role in phagocyte

oxygen metabolism as the electron source for the reduction of superoxide and the intermediate electron donor for the ultimate reduction of hydrogen peroxide.[5]

MECHANISMS OF ENZYME RELEASE: Neutrophils of most species, with the exception of ruminants (see part II), contain two subtypes of cytoplasmic granules. The first granules formed are termed primary or azurophil granules, and the second type formed are secondary or specific granules. These granule subtypes are the stores of inactive enzymes, with the enzymic components varying widely among species.[1,2,4,34,71] Enzymes present within human neutrophil granules have been well defined and are listed in table 2.[34] Enzyme subtypes limited to a particular granule are often used as "markers" of enzyme release or secretion, and they are definable biochemical entities based on their substrates.[1,34,53,54]

The release of enzyme products in their activated forms affords the potential for localized tissue injury. These products may be released or secreted by dying or activated phagocytic cells in response to injury or during the processes of motility and phagocytosis.[1,34] Of the principal granule subtypes, the primary (azurophil) granules have the characteristics of primary lysosomes and contain principally acid hydrolytic enzymes but also peroxidase and neutral proteases.[1] The principal function of these

Table 2. Subcellular Localization of Enzymes and Other Constituents Released by Human Neutrophils

Class of Constituents	Primary (Azurophil) Granules	Secondary (Specific) Granules
Microbiocidal enzymes	Myeloperoxidase Lysozyme	Lysozyme
Neutral serine proteinases	Elastase Cathepsin G Proteinase 3	
Metalloproteinases	Collagenase	Collagenase*
Acid Hydrolases	N-acetyl-B-glucosaminidase Cathepsin B Cathepsin D B-Glucuronidase B-Glycerophosphatase a-mannosidase	
Other		Lactoferrin Vit B_{12}-binding proteins Cytochrome b

*Secondary granule collagenase is released as a latent enzyme.

From Gallin, Reference 34.

granules is the fusion with phagocytic, pinocytic, or autophagic vacuoles, rather than extracellular secretion. The specific granules, however, are particularly accessible for fusion with the plasma membrane, with release of their contents to the extracellular environment. The disposition of specific granule subtypes has led to the use of the term "secretory organs of inflammation".[34] A summary of stimuli which may induce the secretion of primary (azurophil) granules and of secondary (specific) granules is included in table 3.[34]

Exposure of neutrophils to PMA induces a selective loss of specific granules, but no loss of primary granules. Increased levels of intracellular Ca^{2+}, compounds such as concanavalin A and calcium ionophore (A-23187), and cellular adherence or exudation also appear to induce selective discharge of specific granules. Products contained in the specific granules generate the chemoattractant C5a by direct cleavage of C5 and by activation of the complement system.[34] Roles in enzyme secretion have been ascribed for cyclic nucleotides, principally GMP, microtubules, microfilaments, and calcium, which have interrelated functions in assembly of microtubules and microfilaments; and in their interaction with actin and myosin which is necessary for the migration of granules to the plasma membrane, to phagosomes, and for cell motility.[1,4,72] Secretion appears to be cyclic GMP-dependent and requires glycolysis[1] to

Table 3. Stimuli for Granule Exocytosis by Normal Human Neutrophils.

Stimulus	Primary (Azurophil) Granule Exocytosis	Secondary (Specific) Granule Exocytosis
Nonspecific adherence to surfaces	±	+ +
Chemoattractants		
In suspension	-	+ +
+ Surface contact	±	+ +
Pyrogen (interleukin 1)	-	+ + +
Diapedesis (in vivo)	-	+ +
Calcium ionophore A23187	+	+ + +
Phorbol myristate acetate (PMA)	-	+ + +
Lectin (Concanavalin A)	-	+ +
Calcium-dependent spontaneous exocytosis	-	+
Warming to 37 C	-	+
Interaction with opsonized non-phagocytosable surface	+ + +	+
Phagocytosis	+ +	+ + +
Bacterial toxin (eg. Leukocidin)	+ + +	+ + +

*Number of +'s is an estimate of the relative amount of constituent released

From Gallin, Reference 34.

supply the energy requirements. In some instances, adhesion of neutrophils to a surface appears to be necessary for granule secretion.[34] The process of "frustrated phagocytosis", in which neutrophils attempt to surround an object too large to ingest, may also facilitate enzyme release. Regurgitation during feeding, where enzyme contents escape prior to the closing of phagosomes represents yet another mechanism of enzyme release. Soluble factors including complement factors (C5a), formylated oligopeptides (FMLP), and endotoxin may also facilitate direct enzyme secretion.[1,34] As with other processes, degranulation alone cannot be easily separated from concomitant activation of oxidative metabolism or induction of cell motility since these pathways are intimately related.

THE ROLE OF ENDOTOXIN IN NEUTROPHIL FUNCTION: In contrast to the extensive research exploring neutrophil oxidative metabolism, reports of the effects of endotoxin on neutrophil behavior and metabolism are scant. The biological effects of endotoxin are derived from its two major components, lipid A, and the attached polysaccharide chains.[73] The percentages of lipid A, polysaccharides, and the aggregate size of the lipopolysaccharide molecule have been demonstrated to effect variable responses in neutrophils. Those responses will be defined further in this section.

The first major review of the effects of endotoxin on neutrophils, *in vivo* and *in vitro*, was by Morrison

and Ulevitch.[73] The authors attempted to define the mechanism(s) of the profound leukopenia and secondary leukocytosis that is characteristic of *in vivo* endotoxemia. Neutrophil kinetic studies which defined increased release from bone marrow pools, and a shift of granulocytes from the circulating to the marginal pool, afforded a partial explanation of the endotoxin effect. The role of complement factors was studied in experiments that used laboratory animals deficient in complement factors and those depleted of complement by use of cobra venom. All had the same responses to intravenously administered endotoxin[73], implying that complement factors had virtually no role in this response to endotoxemia. Haslett et. al.[74] using ^{111}Indium-labeled neutrophils which were exposed to endotoxin *in vivo* and *ex vivo* has implicated the process of pulmonary sequestration as due to a primary effect on the neutrophil, and independent of complement. The ^{111}In-labeled cells, exposed to endotoxin *ex vivo*, marginated within pulmonary capillaries upon being returned to the circulation without an accompanying systemic leukopenia. In contrast, all cells, labeled and unlabeled, marginated when exposed to endotoxin *in vivo*. The authors were unable to demonstrate any evidence of complement activation on the plasma membranes of treated neutrophils. It was not possible to define the specific product of neutrophils produced or released in response to

endotoxin exposure, although several authors have postulated that lactoferrin, contained within specific granules, may have a role in cell attachment (sequestration). The prolonged attachment of neutrophils to the surface of endothelial cells has been suggested to facilitate cell damage perhaps through the release of reactive oxygen species. However, Hoffstein et. al.[75] determined that contact of neutrophils with a surface actually diminishes superoxide production in response to soluble stimuli.

The *in vitro* responses of neutrophils to endotoxin have been previously reviewed.[73] Recently the *in vitro* responses of neutrophils isolated by differing methods, and/or those exposed to trace quantities of endotoxin were studied by Haslett et. al.[76] Parameters examined included: 1) shape change, 2) chemotaxis, 3) superoxide production, and 4) enzyme release. Spontaneous shape change was enhanced by the use of Ficoll-hypaque isolation techniques, an effect similar to responses to trace endotoxin quantities, and was attributed to the presence of contaminating endotoxin in Ficoll-hypaque. Chemotaxis was markedly reduced, in endotoxin-exposed cells and in those isolated by Ficoll-hypaque techniques, due to increased "stickiness" of the cells. Endotoxin-incubated cells, in response to FMLP, produced more superoxide and released more lysozyme and myeloperoxidase as did cells isolated by

the use of Ficoll-hypaque techniques. There were no significant increases in LDH released by the treated cells, suggesting that the levels of endotoxin used were not directly injurious to the neutrophils.

Guthrie et. al.[77] determined that neutrophils exposed to endotoxin released increased quantities of superoxide and described the effect as "priming". Incubation of cells with trace levels (< 100 ng) of endotoxin stimulated oxygen consumption and the release of superoxide and hydrogen peroxide when compared to unexposed cells. This was indicative of increased respiratory burst activity. For this effect, the neutrophils were incubated in serum-free media for 30 minutes, optimally 1 hour, at 37 C. Other investigators used higher concentrations of endotoxin (5-500 ug), and reported that endotoxin directly stimulated HMP shunt activity[78-80] and increased NBT reduction.[79-81] Lipid A alone (5 ug/ml) increased adherence and superoxide production which was dependent upon cell adherence.[82,83] When particulate fractions of cells were used, there were no increases in superoxide production,[78,79,83,84] chemiluminescence,[80] or NADPH-oxidizing activity.[79] The presence of serum or plasma markedly modified the oxidative metabolism of neutrophils (*in vitro*) through complement activation.[79,80,82] The molecular basis of the "priming" effect was suggested to be: (a) alterations of the plasma membrane so that contact or binding of stimuli might be

greater; (b) events which couple receptor-stimulus interaction to the stimulation of the enzyme necessary for superoxide production (NADPH oxidase) might be altered so that more enzyme is activated; or (c) the conformation or placement of the enzyme in the membrane might be changed so that it expresses higher activity. Since it has been shown that neutrophils have the ability to metabolize endotoxin, it is possible that the lipid A fraction is metabolized to fatty acids which themselves can induce superoxide release and directly activate protein kinase C.[77] The time and temperature requirements were consistent with a metabolite that might directly activate PKC or stimulate increased polyphosphoinositide metabolism, and turnover, indirectly activating PKC.[20]

Dehinden, et. al.[84] used salt forms of a variety of different endotoxins and salt forms of lipid A in assessing granulocyte activation. Endotoxin increased cell adhesiveness and decreased locomotion, whereas respiratory burst activity as measured by increased HMP activity and enzyme release was enhanced. Interestingly, endotoxin appeared to stimulate a selective release of specific granule enzymes but not primary granule enzymes. These responses were independent of the polysaccharide chains of the LPS molecules. Indomethacin failed to alter endotoxin-induced stimulation which suggested independence from products of cyclooxygenase metabolism.

Wilson[85], in his review of the effects of endotoxin on neutrophil function, and Morrison[86] concluded that the marked differences in cell responses, between laboratories, and often between experiments, were primary effects of not only the animal species studied, but also the endotoxin serotype. Since many of the effects of endotoxin on neutrophils are due to complement activation, and are therefore indirect, both authors implied the importance of differentiating between indirect and direct effects. The direct binding of endotoxin to neutrophils has been demonstrated, but the nature of the endotoxin receptor is unclear. Variables which may influence endotoxin binding studies include the bacterial species from which endotoxin is derived, the method of extraction, and the total dose and route of administration. The endotoxin binding and subsequent response of cells is highly dependent upon the aggregate size of the LPS molecule, with the larger forms having more profound effects.[87] Therefore, interaction may occur through either phagocytosis or pinocytosis of the lipopolysaccharide molecule.[88] It was demonstrated previously[82] that exposure of neutrophils to endotoxin markedly enhances adherence and was correlated with the content of lipid A. Lower aggregate forms of LPS were also more potent in increasing cell adherence.[82] Alterations in the surface charge of cells with reduction of the negative surface charge facilitating both adhesiveness and aggregation were determined to be

important factors in adherence.[89] The effect of endotoxin on chemotaxis and random migration, is closely related to increased adhesiveness, and has been termed "cross--deactivation".[84] This effect has been postulated, in part, to be mediated by the release of specific granule substances, possibly lactoferrin.[90,91]

In addition to adverse effects on neutrophil motility, endotoxin also has significant effects on the degranulation responses of exposed cells. When neutrophils were allowed to adhere to a surface, the cells selectively released specific granule components, but not primary granules.[82,84] Some authors have suggested that complement activation is required for granule discharge.[87] High molecular weight LPS aggregates are apparently more efficient at promoting enzyme release.[87] In addition to the effects on enzyme release/secretion, results of other studies have suggested that endotoxin may interact with and directly modify the antimicrobial effects of cationic proteins from neutrophil granules, further increasing the pathogenicity of endotoxin-producing bacterial species.[92]

The effects of endotoxin on neutrophil oxidative metabolism have been categorized as: 1) enhanced killing, 2) increased phagocytosis, and 3) increased HMP activity and NBT reduction. There are marked discrepancies between investigators as to the mechanisms involved, with some reports describing augmentation of responses, and others inhibition.

The necessity for serum in some instances supports a role for the indirect effects of complement factors in these responses. Several reports, previously addressed, have also detailed the apparent requirement for adhesion in the production of increased levels of superoxide and in increased HMP activity, obviously reflecting upon the ability of endotoxin to promote adherence. Apparently, relatively low levels of endotoxin (ng quantities), equivalent to the demonstrated circulating levels in endotoxemia, may produce decidedly different responses than the relatively high levels (ug quantities) used in some of these experiments. In other types of receptor-mediated systems, the affinity state and degree of occupancy of receptors often determine the cell response. The use of trace levels of endotoxin may produce a "priming" effect that is not dependent upon surface contact. This effect, characterized by increased superoxide production and enzyme release occurs in response to such diverse stimuli as immune complexes, opsonized zymosan, and phorbol ester.[93]

LITERATURE REVIEW: PART II. THE BOVINE NEUTROPHIL

INTRODUCTION: When compared to the voluminous numbers of publications and research dealing with neutrophil cellular biology in general, studies dealing specifically with the bovine neutrophil are few. This is particularly true in regards to oxidative metabolism and enzyme release, although other aspects of neutrophil function have been studied. The techniques and procedures used in an attempt to understand the biology of the bovine neutrophil are comparatively primitive in scope, nature, and definition.

BOVINE GRANULOCYTE KINETICS: The intravascular kinetic values for granulocytes of developing calves were determined using ^{51}Cr labeling of neutrophils.[94] Younger animals had a larger total blood granulocyte pool (TBGP) and a shorter intravascular half life ($T^{1/2}$) of circulating granulocytes. As the calves age, the TBGP decreases and the circulating granulocyte pool (CGP) and the $T^{1/2}$ increases. The granulocyte turnover rate in calves 8-16 days of age was $39.91 \pm 6.74 \times 10^8$ granulocytes per kg of body weight per day, with a $T^{1/2}$ of 5.18 ± 0.4 hours.

The large TBGP in neonatal calves is related to their proportionately large blood volume (94 ml/kg.). Age-related changes in blood volume are marked in cattle, but occur in all species.[95] Calves at a young age also have a higher percentage of circulating granulocytes than do older calves or young adults.[96,97]

BIOCHEMICAL CONSTITUENTS OF BOVINE NEUTROPHILS: COMPARISON TO OTHER SPECIES: Phylogenetic comparisons of enzymic components of the neutrophils (heterophils) of several species have been reported by Rausch and Moore (table 4)[98] although no attempts were made to localize these enzymes to either azurophil or specific granule subtypes. The biochemical components of bovine neutrophils were compared to human neutrophils by Gennaro et. al. in 1978 (Table 5)[99]. In these studies, the marker enzyme for bovine neutrophil azurophil (primary) granules was defined as beta-glucuronidase, and for secondary (specific) granules as vitamin B_{12}-binding protein. Compared to human neutrophils, bovine neutrophils were low in primary granule components, high in specific granule components, contained virtually no lysozyme, and had relatively low levels of catalase. The low catalase levels were counterbalanced by higher specific activities of glutathione peroxidase and glutathione reductase. The exposure of the bovine cells to bacteria markedly stimulated increased oxygen consumption, superoxide and hydrogen peroxide production, and glucose

Table 4. Enzyme Activity in PMNs from Various Species.

Species	No. Tested	Units* per 5 X 10^6 PMN ± SEM BGU	MPO	LZM	AKP
Primates:					
Human	7	7.7 ± 2.4	109 ± 44	86 ± 11	11 ± 4
Rhesus Monkey	7	3.0 ± 1.3	38 ± 7	<1	<1
Cynomolgus Monkey	5	0.3 ± 0.1	68 ± 5	<1	114 ± 30
Squirrel Monkey	3**	0.4 ± 0.1	127 ± 9	<1	87 ± 4
Ungulata:					
Cow	4	0.4 ± 0.2	27 ± 2	<1	38 ± 8
Goat	9	0.6 ± 0.1	6 ± 1	<1	27 ± 11
Sheep	9	1.1 ± 0.4	19 ± 3	<1	30 ± 4
Horse	3	1.0 ± 0.3	39 ± 10	16 ± 1	471 ± 76
Burro	11	1.7 ± 0.4	63 ± 9	8 ± 1	536 ± 201
Carnivora:					
Cat	3	2.9 ± 0.7	9 ± 1	<1	<1
Dog	6	0.6 ± 0.2	95 ± 26	9 ± 2	8 ± 4
Lagomorph:					
Rabbit	5	0.7 ± 0.2	13 ± 6	15 ± 3	99 ± 46
Rodentia:					
Rat	10**	5.1 ± 0.6	46 ± 4	10 ± 2	95 ± 34
Guinea Pig	6	2.1 ± 0.2	11 ± 3	15 ± 3	471 ± 91
Mouse-AKR	2**	0.7	18	10	<1
Mouse C57	2**	1.0	20	5	<1
Hamster	3**	0.7 ± 0.1	16 ± 3	<1	273 ± 23
Aves:					
Chicken	3	1.1 ± 0.5	0	84 ± 14	<1
Goose	4	0.9 ± 0.1	0	24 ± 4	<1

PMN=polymorphonuclear leukocyte; BGU=beta glucuronidase; MPO=myeloperoxidase; LZM=lysozyme; AKP=alkaline phosphatase; *BGU and AKP, nmole/min; MPO change in OD/min (X 10^3); LZM, ug.; **Number of pools tested. From Rausch and Moore, reference 98.

Table 5. Enzyme Activities and Content of Binding Proteins of Granulocytes of Bovine and Human Blood[a]

Enzymes and binding prots.	Bovine granulocytes	Human granulocytes
1. B-Glucuronidase	12.8 ± 0.3 (8)	202.1 ± 18.0 (3)
2. B-Galactosidase	11.8 ± 0.6 (9)	62.4 ± 7.4 (3)
3. Myeloperoxidase	1.6 ± 0.1 (8)	4.7 ± 0.1 (3)
4. Acid phosphatase	36.7 ± 6.3 (5)	—
5. Alkaline phosphatase	230 ± 9 (8)	23.9 ± 7.0 (3)
6. Lysozyme	< 0.2 (3)	31.8 ± 3.6 (3)
7. Lactoferrin	74 ± 7 (7)	30-64(Refs in art)
8. Vitamin B_{12}-binding prot.	26.7 ± 2.8 (3)	4-9(Refs in art)
9. Malate dehydrogenase	0.49 ± 0.04 (6)	—
10. Catalase	7.1 ± 0.5 (3)	108.2 ± 11.1 (4)
11. Glutathione peroxidase	14.1 ± 0.6 (4)	5.2 ± 0.4 (4)
12. Glutathione reductase	25.0 ± 0.8 (4)	13.2 ± 1.1 (4)
13. Protein	1.21 ± 0.03 (14)	—

[a]Sixteen preparations of bovine granulocytes, and four of human granulocytes; The bovine cells had 92.1% ± 0.4% neutrophils, 5.3% ± .3% eosinophils, 2.6% ± 0.1% monocytes; Data in means ± SEM/10^7 cells; Number of experiments in brackets; 1 & 2: nmoles methylumbelliferone/30 min; 3: umoles t-guiacol/min; 4 & 5: nmoles p-nitrophenol/min; 6 & 7: ug; 8: ng vit B_{12}; 9: umoles NADH/min; 10: umoles H_2O_2/min; 11 & 12: nmoles NADPH/min; 13: mg. Refs in art=references in article. From Gennaro et. al. reference 99.

oxidation through the HMP pathway. Bovine neutrophils also had the ability to kill ingested staphylococci under conditions in which the organisms were not killed by human neutrophils. In bovine cells, the catabolism of hydrogen peroxide was principally through the glutathione system which coincided with the marked HMP activation due to increased generation of NADP.

In 1983 and 1985, Gennaro and co-workers identified and defined a third granule subtype in bovine neutrophils, and subsequently in other ruminant species.[100,101] These granules, were found to constitute the largest proportion of intracytoplasmic granules, and were formed during myelopoesis after the primary granules, but before the secondary granules. By reaction of cells with peroxidase, these granule subtypes could be differentiated ultrastructurally. Biochemically, the granules were found to consist principally of cationic bactericidal proteins. Further studies revealed that these granules were secreted independently from primary and secondary granules. The major role of this third granule subtype was in oxygen-independent killing of bacterial organisms. Unfortunately, there is at present no defined biochemical marker for these granules. Therefore, their roles in relation to host response and protection against bacterial pathogens remains uncertain.

BOVINE NEUTROPHIL FUNCTION STUDIES: To adequately study neutrophil function specialized techniques are

required to isolate bovine neutrophils from the other components of peripheral blood. The technique described by Roth and Kaeberle[102], differential centrifugation and hypotonic lysis, provides the highest yield and purity of neutrophils although several other methods have been described.[103-106] The yield of neutrophils is age-dependent since young calves have higher total white blood cell (WBC) counts and a higher percentage of neutrophils.[96,97] Interpretation of data from function studies in older animals is often complicated by high levels of eosinophils that possess diametrically different behavioral responses when compared to neutrophils.

The majority of research papers describing the varied aspects of bovine neutrophil function have been authored by Roth, Kaeberle, and colleagues. In a pioneering paper published in 1981[102] they described five in vitro techniques for evaluation of PMN function which included: 1) random migration under agarose; 2) ingestion of ^{131}I-iododeoxyuridine labeled *Staphylococcus aureus* (SI); 3) quantitative nitroblue tetrazolium (NBT) reduction; 4) chemiluminescence (CL); and 5) iodination. In more recent publications, antibody-dependent-cellular cytotoxicity (ADCC) has also frequently been used to assess neutrophil function.

In studies exploring the effect of bovine viral diarrhea (BVD) virus on neutrophils[107], the uptake of an this virus has the capability to impair the

emulsion of paraffin oil and *Escherichia coli* lipopolysaccharide (LPS), NBT reduction, CL, and iodination were used. Only iodination was significantly reduced, suggesting that myeloperoxidase-halide-hydrogen peroxide antibacterial system. Administration of a vaccinal strain of BVD virus[108] alone produced suppression of neutrophil iodination and ADCC. When given concurrently with ACTH, there was enhanced random migration, enhanced SI, with suppressed iodination and ADCC. The neutrophils of cattle persistently infected with BVD virus[109] had a decreased capability to ingest staphylococci, but had normal random migration under agarose, cytochrome C reduction, iodination, and ADCC. These responses were significantly different from animals mounting an immune response to acute infection and from the responses to vaccinal strains.

The effects of stress on neutrophils were mimicked by the administration of ACTH[110] which enhanced random migration, did not alter SI, NBT, or ADCC and markedly reduced iodination. These changes in neutrophil responses were closely related to increased levels of serum cortisol. In a companion study[111], the administration of a single dose of dexamethasone duplicated the previous findings but in addition also depressed CL. In an experiment to determine the effects of ascorbic acid on healthy and dexamethasone-treated cattle[112], ascorbic acid alone enhanced oxidative

metabolism and ADCC, with no effects on random migration, SI, or iodination. Dexamethasone increased random migration and depressed all other functions. The administration of ascorbic acid reversed the effects of dexamethasone on all functions in a dose-dependent manner.

Random migration, SI, NBT, iodination, and ADCC were used to assess the effects of estradiol and progesterone on neutrophil function in steers.[113] Estradiol had no effect on neutrophil function, whereas progesterone depressed iodination and enhanced random migration, with no significant difference in SI, NBT reduction or ADCC.

Several studies explored the effects of bacterial organisms on neutrophil function. When the effects of capsulated vs noncapsulated forms of *Pasteurella multocida* were compared, the presence of live organisms, heat-killed whole cells and capsular extracts inhibited both SI and iodination, whereas there was no effect on NBT reduction.[114] Results of studies using *Haemophilus somnus*[115] revealed that live, heat-killed, or supernatants from heat-killed cells inhibited iodination and SI, whereas heat-killed and washed cells did not. The factor which prevented SI was a high molecular weight substance (300,000 MW) while the factor inhibiting iodination was under 10,000 MW. Further studies[116] defined the low molecular weight factors as ribonucleotides and purine and pyrimidine bases, with guanine and adenine the principal inhibitors.

Czuprynski and Hamilton[117] also reported that the ingestion of *Haemophilus* required opsonizing antibodies, but the cells were unable to kill the organisms. The ingestion of the organisms by neutrophils stimulated a minimal CL response, perhaps due to the inhibitory effects of guanine and adenine as defined by Roth et. al.[115,116] The capacity of *Brucella abortus*[118] to survive intracellularly was investigated and revealed a marked decline in iodination and preferential inhibition of primary granule degranulation upon exposure to a low molecular weight extract of the organism. Further experiments[119] were designed to explore these effects. Neither SI or NBT reduction was inhibited by heat-killed *Brucella abortus*. The ability of PMNs to iodinate proteins was inhibited by live or heat-killed *B. abortus*, and supernatants from heat killed cells but not by washed heat-killed cells. Inhibitory components were characterized as nucleotide-like substances with molecular weights of 1000. This inhibition was concentration dependent, and suggested that the organism may escape killing by the production of these inhibitory substances. The substances were subsequently determined to be GMP and adenine, and their production by the organism contributed to this inhibition.[120]

Studies of the comparative effects of recombitant gamma interferon and an antigen-induced lymphokine on neutrophils[121] revealed decreased migration under agarose and

increased ADCC with no effect on SI or iodination. The lymphokine increased cytochrome c reduction and was mildly chemotactic, but the recombitant form had no similar effects. The interferon acted as a migration inhibitory factor and as a neutrophil activation factor. A specific protocol[122] evaluated the effects of an antigen-induced lymphokine on antibody-independent neutrophil cytotoxicity (AINC). The lymphokine caused inhibition of neutrophil migration and enhanced adherence. The compound also enhanced NBT reduction, SI, and ADCC. The lymphokine-treated cells became cytotoxic for chicken, turkey, and human erythrocytes but not for bovine erythrocytes. The increased AINC was not due to cytotoxicity of the lymphokine or to mediator release by the neutrophils, but required neutrophil protein synthesis. This indicated that neutrophils could be induced to recognize and increase cytotoxic activity to heterologous erythrocytes. Reports by Ohmann and Babiuk[123] confirmed the inhibition of random and directed migration in response to alpha-1 interferon but also described enhanced bacterial uptake, increased enzyme release, increased hydrogen peroxide production, and decreased superoxide release. In a later report[124] a comparison of the gamma and alpha forms showed enhancement by the gamma form in suppression of migration and in the production of superoxide. *In vitro*, both forms suppressed migration and had little effect on superoxide production except at extremely high concentrations.

In a paired study,[125,126] thiabendazole was used in an attempt to improve the immune response of dexamethasone-treated or stressed cattle. It was determined that the drug had no effect on the response to administered antigens and significantly lowered the antibody response to *B. abortus*.[125] The compound had no effect on neutrophil function parameters, such as random migration, NBT reduction, iodination, or ADCC. There were significant effects on lymphocyte blastogenic responsiveness, tending to normalize responses after dexamethasone administration.[126]

Since it has been reported that neonates of all species are more susceptible to infectious processes, in part due to defective neutrophil function, age-related changes in SI, NBT reduction, iodination, and ADCC were studied.[127] Four groups of calves were used with age groups of: 4-5 weeks, 9-11 weeks, 16-19 weeks, and 12-14 months. Iodination in the two youngest age groups was approximately 50 % of the older groups. ADCC was much lower in the three youngest age groups, whereas NBT reduction was lowest in the youngest group only. SI capability was higher in the three younger groups versus the older group. The authors concluded that the differences in cell function might partially explain the increased susceptibility of young calves to disease.

Of principal interest in this dissertation is the role of products of neutrophil metabolism in the pathogenesis of

pneumonic pasteurellosis of ruminants. The report of Slocombe et. al.[128], which used calves depleted of neutrophils by hydroxyurea administration,[129] determined the necessity for neutrophils in this disease, Calves with normal numbers of circulating neutrophils developed hypoxemia, tachypnea, bradycardia, neutropenia, and lymphopenia, in response to intratracheal administration of *Pasteurella haemolytica*. These animals had gross and microscopic lesions characteristic of pneumonic pasteurellosis. Neutrophil-depleted calves did not develop physiologic abnormalities, nor did they develop gross or microscopic pulmonary lesions. They concluded that pneumonic pasteurellosis is a neutrophil-mediated disease.

Several investigators have explored the interaction between *Pasteurella haemolytica* (PH) and neutrophils. Emphasis has been placed upon determining the role of cytotoxin, produced by log phase organisms, in the disease. Berggren et. al.[130] conducted an opsonophagocytic assay using log phase and stationary phase organisms. Using trypan blue exclusion and ^{51}Cr-release, they reported a marked reduction in phagocytosis of log phase organisms and showed that the log phase organisms liberated a soluble factor that was cytotoxic to neutrophils. The cytotoxic factor was heat labile, oxygen stable, and susceptible to extremes of pH, but it was not hemolytic. The substance had a molecular weight of > 300,000, as determined by

ultrafiltration. Czuprynski et. al.[131] determined that ingestion of virulent *Pasteurella* by neutrophils was serum dependent and mediated principally by heat stable opsonins (antibodies). Killing occurred within 1-4 hours of ingestion, with reduced neutrophil viability at bacteria to cell ratios of 100:1. The ingestion of opsonized PH stimulated a marked CL response. O'Brien and Duffus[132] compared the susceptibilities of bovine leukocytes to the PH cytotoxin and determined that neutrophils were the most susceptible, macrophages less so, and that bronchoalveolar macrophages from adult animals were the most resistant. They also determined that sub-cytolytic concentrations of cytotoxin did not impair killing of virus infected cells by macrophages.

Chang et. al.[133] used luminol-dependent chemiluminescence (LDCL) to study the responses of neutrophil preparations from 4 species of ruminants and 6 species of nonruminants to opsonized and nonopsonized live and dead PH and *Staphylococcus aureus*. Opsonized organisms enhanced the reponse in all species. Living PH and culture supernatants inhibited the neutrophil LDCL for all ruminants, but not for nonruminants. In a companion study[134] the LDCL responses of bovine neutrophils to living and killed PH, *Pasteurella multocida*, and *Escherichia coli* were evaluated. Live and opsonized bacteria enhanced responses, but with PH the LDCL precipitously declined after

a peak response at 10 minutes. Similar responses were seen with culture supernatants. The authors suggested that the LDCL-inhibition might be used to quantitate the concentrations of cytotoxins produced by PH. A subsequent report[135] compared the utilization of the LDCL-inhibition (LDCLI) assay to trypan blue dye exclusion (TBDE) and to ^{51}Cr-release assays (CRA) and determined the relative sensitivities of the assays in detecting biological activity of PH leukotoxin. These studies revealed the LDCLI assay to be 17 times more sensitive than the CRA and 2,480 times more sensitive than the TBDE. The authors proposed the utilization of chemiluminescence inhibitory units (CIUs), which were defined as the quantities of cytotoxin required to abbrogate the chemiluminescence response of 10^6 neutrophils within 20 minutes of incubation. Serial dilutions of cytotoxin were used to determine the least amount that would inhibit cell response.

Other investigators have also used the production of chemiluminescence in the study of other parameters of bovine neutrophil function. Forsell[136] used the generation of CL and phagocytosis assays to determine that the wood preservative pentachlorophenol had no effect on neutrophil function. Phillips et. al.[137] investigated the effects of dexamethasone administration on neutrophil CL and found that there were no significant effects on phagocytes in whole blood, but that single or multiple doses of the drug

enhanced the CL response of purified cells. A companion report[138] determined that PGE1, PGE2, indomethacin, and histamine suppressed the CL response and that the suppression was dependent upon the continuous presence of the compounds. However, the inhibition of CL induced by indomethacin persisted even after removal of the compound from the culture media.

Few studies of bovine neutrophil oxidative metabolism have quantitated either hydrogen peroxide or superoxide anion production or measured oxygen consumption, all considered to be measures of respiratory burst activity. Korhonen and Reiter[139] compared the production of H_2O_2 by blood-derived and milk-derived neutrophils. Hydrogen peroxide could not be detected in the presence of bovine serum, fetal calf serum, or milk whey unless sodium azide (NaN_2) was added to antagonize endogenous catalase and lactoperoxidase. Bovine serum or milk whey was required for phagocytosis of live organisms and for hydrogen peroxide production. IgG2 and to a lesser extent IgG1, but not SIgA and IgM, stimulated hydrogen peroxide release independently of phagocytosis. Neutrophils isolated from peripheral blood were more active in hydrogen peroxide production than were those isolated from milk. In 1986, Young and Beswick[140] compared the responses of bovine, porcine, ovine and human neutrophils to serum-treated zymosan and found significantly less superoxide production and oxygen

consumption of animal species compared to human cells. The differences in responses did not appear to be influenced by the origin of the serum. Human neutrophils responded to FMLP, but the neutrophils of the animal species did not.

THE ROLE OF OPSONINS IN BOVINE NEUTROPHIL RESPONSES: The quantities of opsonins in serum are of major importance in the facilitation of phagocytosis and involve the levels of specific immunoglobulins,[141-144] as well as products of the complement cascade pathways.[145-148] Complement products such as C3b, derived from the classical pathway, and C5a, derived from alternate and classical pathways, are important not only in their roles as chemotaxins, but when deposited on a surface or particulate entity they enhance neutrophil ingestion through the interaction with cell surface receptors. While the pathways of complement activation and their role in the process of opsonization are well understood, the participation of bovine immunoglobulin subclasses in this process remains a subject of confusion and debate. Principal roles for IgG1, IgG2, and IgM in the fixing of complement and the enhancement of phagocytosis have been described by several investigators.[141-143] In neonatal calves, early protection against infection is derived from the absorption of colostral immunoglobulins with IgG1 being preferentially absorbed over IgG2 by an approximate ratio of 7:1.[141] While both IgG1 and IgG2 can fix complement by means of the classical pathway, it was determined

that only IgG2 fixes it by means of the alternate pathway[141,143] and facilitates phagocytosis.[143] Since the defense against gram negative organisms, such as *Pasteurella*, is principally dependent upon the alternate pathway, selective deficiencies of either IgG2 or alternate pathway components might render neonatal calves more susceptible to bacterial disease. While immunoglobulins are passively acquired, complement factors are not, and normal (adult) levels are not reached until approximately six months of age.[145,148]

Lombardo et. al.[149] compared the effects of colostral ingestion on leukocyte counts, and found that calves fed colostrum had higher total leukocyte counts and higher percentages of neutrophils than did colostrum-deprived calves. For functional studies of bacterial ingestion by neutrophils, *E. coli* organisms were opsonized with three serums: 1) neonatal pre-colostral, 2) autologous post-colostral and 3) adult, with monitoring of neutrophil responses by quantifying oxygen uptake. The highest oxygen uptake rate was obtained by the use of adult serum, with autologous intermediate, and neonatal the lowest. The use of neonatal serum for opsonization produced no significant differences in the responses of neutrophils derived from colostrum-fed (CF) and colostrum-deprived (CD) calves. However, the use of autologous or adult sera significantly increased oxygen uptake. Activity of the HMP shunt and glycolysis

were examined by determining the oxidation of labeled ^{14}C-glucose to CO_2 and lactate. There were no significant differences between CF and CD in HMP activity or in lactate formation.

The effects of maternal protein-energy malnutrition and cold stress on neutrophil function and sera were studied by Woodard et. al.[150] Nutritional deficiencies in the dam had little effect on *in vitro* bactericidal activity of neutrophils or pre-colostral sera taken at birth. Neutrophils obtained at birth killed *Staphylococcus aureus* but not *Escherichia coli*, when incubated with heated or unheated autologous serum. With pre-colostral serum, neutrophils from 3-day-old calves were no more active in destroying bacteria than those of newborn calves. The addition of day 3 serum enabled day 3 neutrophils to destroy *E. coli*, but this was not influenced by heat treatment of sera. Maternal protein deficiency increased the destruction of *E. coli* by day 3 neutrophils and sera. There were no differences in the bactericidal effects of neutrophils or sera from cold stressed calves, nor were cold stress-nutritional stress interactions detected.

SELENIUM AND VITAMIN E AND NEUTROPHIL FUNCTION: The necessity for both of these compounds as major components of intracytoplasmic and membranous defense against peroxidative attack from oxygen and lipid derived radical species, has been addressed in Part I of this review. At high

levels of supplementation vitamin E enhances both humoral and cellular immunity by means of an, as yet undefined, enhancement of the phagocytosis of antigens.[66] Adequate selenium also appears to be necessary for humoral response.[151] Selenium deficiencies[152,153-156] while not affecting the ability of neutrophils to phagocytize yeasts (*Candida albicans*)[155] or bacteria (*Staphylococcus aureus*)[152,157], markedly diminish the ability of cells to kill ingested organisms.[157] In neutrophils, the production of superoxide and hydrogen peroxide is initiated by activation of the respiratory burst enzyme NADPH-oxidase in which the rate limiting step is the availability of reducing equivalents (NADP) supplied by the activation of the HMP shunt. Sources of NADP include the superoxide forming reaction and catabolism of hydrogen peroxide by means of the glutathione reductase and glutathione peroxidase system. Since selenium is required for activity of the selenium-dependent form of glutathione peroxidase, and since this enzyme represents the primary means of disposal of hydrogen peroxide in bovine neutrophils[99], impaired function limits the production of NADP, reducing the generation of superoxide, and thereby altering killing ability.[152]

ENDOTOXIN AND BOVINE NEUTROPHILS: The principal *in vivo* studies in the bovine species have used the intramammary administration of endotoxin to determine the effects of and similarities to naturally occurring endotoxic

mastitis. Endotoxin administration induced significant increases in milk whey IgG1, IgG2, IgM, and IgA with the greatest relative increases seen for IgG2. Cows with endotoxin-infused quarters, treated systemically with flunixin meglumine, had significantly decreased milk whey concentrations of IgG1 and IgM. While endotoxin treated quarters seemed to enhance the phagocytosis of *Staphylococci* by milk PMNs, there were no differences in the flunixin or saline treated (control) animals.[158] In mastitis, neutrophil influx is immunoglobulin related, but few studies have attempted to determine whether endotoxin has direct effects on the bovine neutrophil. One study determined that a principal effect of LPS was the inhibition of cellular migration. In the only report dealing with the direct, *in vitro* effects of LPS on neutrophils, Confer and Simons[159] used *Pasteurella haemolytica* LPS and determined that PH LPS itself was not toxic to neutrophils, and had little effect on neutrophil random migration. Phagocytosis of labeled *Staphylococcus aureus* was decreased by low (2.5 ug/10^6 cells) and high (65 ug/10^6 cells) concentrations of LPS, whereas moderate levels (5-25 ug/10^6 cells) increased the rate of ingestion. PH LPS also enhanced the NBT reduction of exposed cells.

OVERVIEW AND CONCLUSIONS: Part I of this review consisted of an attempt to summarize, in a concise manner, the current thought and perspectives of neutrophil cellular

biology and mechanisms of transduction. While the aspects that pertain to the major postulated mechanisms of neutrophil-mediated injury: 1) products of oxidative metabolism, 2) enzyme release, and 3) release of arachidonate metabolites have changed little over the years, the increased sophistication of research efforts, which has enabled investigators to dissect cellular responses, has led to an increased level of understanding of the complexity of the biochemical events leading to neutrophil-mediated tissue injury. This often overwhelming mountain of information on the cellular biology of neutrophils have been a result of the desire by all investigators to determine the solitary mechanism for each response, the mechanistic approach to investigation. Neutrophils, however, are a biological match for the appetites of these micromolecular investigators, in that no solitary stimulus induces a solitary response. In contrast to the mechanistic methods of part I, studies of the biology of the bovine neutrophil are functionalistic. Rather than attempting to define the mechanism of a response, they have sought instead to define the response. In comparison to the techniques used in Part I, many of our techniques are comparatively primitive in nature. Only time will tell whether the mechanistic approach or functionalistic approach will provide the ultimate benefits to the species we have chosen to study.

In subsequent chapters, I will describe the results of my research into the role of neutrophil products in pneumonic pasteurellosis of ruminants. These experiments were designed to determine: 1) whether products of bovine neutrophil oxidative metabolism might have a role in the pathogenesis of the disease, 2) whether bacterial lipopolysaccharide (endotoxin) had effects on bovine neutrophil oxidative metabolism that might contribute to the pathogenesis of the disease, and 3) whether the release of cytoplasmic granules and enzymes by stimulated neutrophils might have a role in the pathogenesis of the disease.

In Chapter 1, I will describe the means and methods used to define the oxidative metabolism of bovine neutrophils. For these experiments I chose to use chemiluminescence, enhanced by luminol, (LECL) and superoxide production to quantitate the neutrophil responses. To provide a basis for future comparisons of bovine neutrophil oxidative responses, I chose to use a series of agonists which induce neutrophil oxidative metabolism by different mechanisms. To ensure that the concentrations or quantities of these agonists maximally activated bovine neutrophils a series of dose response studies was intitially performed. The results of these experiments led to the definition of a battery of agonists which were subsequently used in comparisons of the oxidative responses of neutrophils to the *Pasteurella* organism. The spectrum and concentrations

of "defined agonists" were used in later experiments to determine the effects of endotoxin on oxidative responses of bovine neutrophils and in studies defining enzyme release.

In Chapter 2, interest into the possible effects of endotoxin on neutrophil oxidative metabolism was fueled by a recent report of increased superoxide production after exposure of neutrophils to trace quantities of endotoxin.[77] I questioned whether endotoxin might have similar effects on bovine neutrophils. To answer this question I used trace quantities of two different endotoxins, *E. coli* O55:B5 endotoxin and *P. haemolytica* endotoxin. I sought to resolve the premise that the biological effects of endotoxins differ little between bacterial species of origin and to determine whether the effects of *Pasteurella* endotoxin might induce a response that was specific for bovine neutrophils. Through the use of the previously defined agonists, I hoped to determine whether endotoxin-induced effects were specific for some mechanisms of induction but not for others.

In Chapter 3, I used the release of B-glucuronidase, a marker of primary granule release, vitamin-B_{12}-binding protein, a marker of secondary granule release, and lactate dehydrogenase, a measure of neutrophil death/lysis, to determine the spectra of enzyme release in response to the previously defined agonists. The results of these studies

provided a basis for comparing the amounts of these markers released by exposure of neutrophils to *P. haemolytica*. I hoped to determine whether the selective release of granules subtypes, induced by the organism, might have a significant role in pneumonic pasteurellosis.

In summary, I hoped to interpolate my understanding of the mechanisms of neutrophil transduction, from experiments suggested by the reports of other investigators, into a series of experiments which would allow me to define the mechanisms of neutrophil-mediated injury induced by the *Pasteurella* organism. In definition of these mechanisms, I hoped to provide a basis for future studies into the modulation of the disease.

CHAPTER 1. LUMINOL ENHANCED CHEMILUMINESCENCE AND SUPEROXIDE PRODUCTION BY BOVINE NEUTROPHILS IN RESPONSE TO SELECTED AGONISTS: COMPARISON OF THE RESPONSES TO *PASTEURELLA HAEMOLYTICA*

Introduction: Neutrophils respond to soluble and particulate agonists by the sequential processes of chemotaxis, phagocytosis, degranulation and increased metabolic ("respiratory burst") activity.[1-3] As a consequence of neutrophil activation, pulmonary injury may occur. Pulmonary injury by neutrophils involves three major mechanisms: the generation of oxygen-derived free radicals, the synthesis and release of proteolytic enzymes from specialized cytoplasmic granules, and liberation of arachidonic acid-derived metabolites.[1-3,5,160-164] No unifying theory adequately explains differences in the mechanisms of neutrophil-mediated injury, although a basic understanding of these processes is known. In some diseases tissue injury is caused by secretion of proteolytic enzymes[165-168], yet in others injury may be lessened by blockers of oxygen radical formation or by inhibitors of arachidonic acid metabolism.[169-176]

The release of oxygen-derived radicals, enzymes and arachidonate metabolites may occur by means of diffusion, secretion, cell lysis, reverse endocytosis ("frustrated phagocytosis"), or by regurgitation during feeding when phagosomal contents escape prior to closing of phagocytic vacuoles.[1] Adverse effects of neutrophils have been demonstrated in acute pulmonary diseases such as pulmonary thromboembolism, endotoxic shock, adult respiratory distress syndrome (ARDS) and immune complex interstitial pneumonias.[177-181] Neutrophil depletion in sheep[182] and cattle[129] has been used to define the role of neutrophils in the mediation of lung injury in endotoxemia and in bacterial pneumonia. Although neutrophils have been incriminated in the initiation of pulmonary injury in pneumonic pasteurellosis[129], there is no information regarding which neutrophil products are damaging in this disease syndrome. Experiments in our laboratory in which calves were pretreated with polyethylene glycol (PEG)-bound catalase to catabolize the release of hydrogen peroxide, or with cyclooxygenase inhibitors did not diminish the severity of experimentally-induced *Pasteurella* pneumonia (unpublished data).

Studies by Gennaro et. al.[99] determined that stimulated bovine neutrophils had a greater capacity to produce superoxide (O_2-) and hydrogen peroxide (H_2O_2) than did human neutrophils responding to the same stimuli. To

further evaluate these responses, two measures of neutrophil oxidative metabolism were chosen: luminol-enhanced chemiluminescence (LECL) and superoxide production. Concentrations of agonists which induced maximal neutrophil oxidative metabolism in other species[53,54] were used as a guide for dose response studies and subsequently in experiments to determine the magnitude of bovine neutrophil LECL and superoxide production. The spectrum of agonists selected to evaluate the responses of bovine neutrophils to differing mechanisms of transduction (activation) included: 1) non-opsonized particulates (latex); 2) opsonized particulates [opsonized zymosan (OZ)]; 3) an exogenous activator of protein kinase C [phorbol myristate acetate (PMA)]; and 4) an inducer of increased intracellular calcium [calcium ionophore, A23187 (CI)].

The increased susceptibility of neonates to disease has been documented and partially ascribed to defective neutrophil function in human infants[183,184] and calves.[127,149,152] A series of experiments was performed to evaluate whether the neutrophils of calves of different ages had differences in LECL or superoxide production responses which might be attributed to induction by specific agonists.

In summary, this series of experiments was designed to: 1) define some of the factors influencing bovine neutrophil oxidative responses, 2) define the concentrations of

agonists which maximally stimulate bovine neutrophil oxidative metabolism, 3) quantitate the magnitude of LECL and O_2- production of bovine neutrophils stimulated by defined agonist concentrations, 4) determine the effects of age on the oxidative metabolism of bovine neutrophils stimulated by defined concentrations of agonists, and 5) compare the oxidative responses stimulated by agonists with those stimulated by *Pasteurella haemolytica*.

MATERIALS AND METHODS:

Experimental Animals: Neonatal Holstein bull calves, less than 1 week of age, were obtained from a large local dairy farm and had received colostrum. On arrival at MSU the calves were weighed, given an oral rotavirus and coronavirus vaccine[a], and injections of vitamin E/selenium[b] and vitamins A, D, and E[c]. Blood samples were collected in EDTA tubes[d] for total and differential white blood cell (WBC) counts. Twice daily, body temperature, pulse and respiratory rates (TPR), appetite, attitude, and character of feces were recorded. The calves were housed in a stall in a research ward of the Veterinary Clinical Center (VCC), fed milk replacer[e] twice daily and exercised once daily. The calves were acclimated for one week before use

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[a]Calf Guard, Norden Laboratories, Lincoln, NE.
[b]Bo-Se, Schering Corporation, Kenilworth, NJ.
[c]Vitamin ADE Injection, W. A. Butler Co., Columbus, OH.
[d]Vacutainer Tubes, Becton-Dickinson, Inc, Rutherford, NJ.
[e]Fresh Start, Vita Plus Coporation, Madison, WI.

and during this time several aliquots of serum were harvested for later use in the opsonization of particulate agonists. Each calf was used for three weeks.

Preparation of Solutions: Hank's balanced salt solution (HBSS) and phosphate buffered saline (PBS) with and without calcium and magnesium were prepared from concentrated stock solutions.[53,54] Triple deionized water (TDW) was phosphate buffered (0.0132M)[102]. All solutions were adjusted to a pH of 7.2 and stored at 4 C. Acetate-citrate-dextrose (ACD) anticoagulant was prepared by the large animal pharmacy of the VCC and kept at 4 C. All solutions were sterilized by filtration prior to storage and prepared as needed.

Neutrophil Isolation: Venous blood was collected in syringes containing ACD (1ml per 6.6 ml of blood), transferred into blood tubes, and placed in crushed ice. (see "Effect of blood handling", Materials and Methods) Total WBCs were counted with an automated cell counter[f], blood smears were stained with a modified Wright-Giemsa stain[g], and manual differential WBC counts were performed prior to neutrophil isolation. Total and differential (% neutrophil) counts were recorded. Procedures for isolation of neutrophils were modifications of previously described techniques.[102-106] Briefly, the blood was centrifuged at

[f]Coulter Counter ZBF, Coulter Electronics, Hialeah, FL.
[g]Camco Quick Stain, Cambridges Chemical Products, Inc., Detroit, MI.

1000 X g for 20 minutes in 16 X 125 mm glass tubes, and the plasma, buffy coat and 1/4 to 1/3 of the red blood cell layers were aspirated. Erythrocytes were lysed by the addition of TDW (two times the cell volume). The tubes were rapidly but gently inverted 20 times, and an equal volume of 2X PBS (without Ca^{2+} and Mg^{2+}) was added to restore isotonicity. The cells were centrifuged at 250 X g for 10 minutes, the supernatant was aspirated, and the cells were resuspended in calcium and magnesium-free HBSS. A second lysis, centrifugation and resuspension were then performed. Total and differential WBC counts and trypan blue dye exclusion tests for viability were performed on the isolated cells. Test tubes containing the isolated cells were stored in crushed ice until used. All assays were performed within six hours of blood collection to ensure maximal cell viability.

Preparation of Agonists and Reagents: Luminol[h] (5-amino-2,3-dihydro- 1,4-phthalazinedione) was prepared fresh weekly in PBS with Ca^{2+} and Mg^{2+} at a stock concentration of 10^{-3} M, adjusted to pH 7.2, and allowed to dark adapt at 4 C for a minimum of 72 hours. Phorbol 12-myristate 13-acetate[h,i] (PMA) was dissolved in dimethylsulfoxide (DMSO) at a concentration of 2 mg per ml, and aliquots were stored at -70 C. Calcium ionophore[h] (CI)

[h]Sigma Chemical Company, St. Louis, MO.
[i]CMC Cancer Research Chemicals, Brewster, NY.

was dissolved in DMSO to obtain a stock solution of 2 X 10^{-2} M and stored at -20 C. Formyl-methionyl-leu-cyl--phenyl-alanine (FMLP)[h] was dissolved in DMSO at a concentration of 10^{-2} M, with aliquots stored at -70 C. Zymosan[h] (10 mg/ml) was suspended in HBSS (with Ca^{2+} and Mg^{2+}), and opsonized by adding an equal amount of serum to the samples which were placed in a shaking water bath[j] at 37 C for 30 minutes. The OZ was washed twice in HBSS and centrifuged at 4 C, with aliquots stored at -20 C. Zymosan-activated serum (ZAS) was prepared by the addition of equal volumes of serum to a zymosan suspension (10 mg/ml) and incubated for 45 minutes in a shaking water bath at 37 C. Post-incubation, the tubes were centrifuged at 1000 X g for 15 minutes at 4 C, and the serum was decanted and heat inactivated at 56 C for 30 minutes. Aliquots were stored at -70 C. Latex particles[h], 0.81 micrometer, were mixed thoroughly in a vortex mixer prior to use. Cytochrome C (Horse heart, type VI)[h] was dissolved in HBSS at 7.5 mg per ml (0.6mM) for immediate use. Superoxide dismutase (SOD)[h,k] was dissolved in HBSS at 1000 units of activity per ml. The enzyme solution was stored at -20 C, and thawed just prior to use.

Stock cultures of *Pasteurella haemolytica* serotype A1 were inoculated into BHI broth or onto blood agar plates

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[j]Dubnoff Metabolic Shaking Incubator, American Scientific Products, McGaw Park, IL.

[k]DDI Pharmaceuticals, Mountain View, CA.

and grown at 37 C for 48 hours. The bacteria in broth tubes were centrifuged (1000 X g), the broth was decanted and discarded, and the bacteria were resuspended in HBSS with $Ca^{2+}$and Mg^{2+} by means of a vortex mixer. The washing process was repeated twice. Bacterial colonies from blood agar plates were removed with a sterile plunger from a tuberculin syringe and by washing with HBSS. The bacterial suspensions were centrifuged at 1000 x g. After washing, the bacterial organisms were resuspended in HBSS and adjusted to an optical density of 0.800 at 541 nm in a double beam spectrophotometer.[1] The bacterial suspensions were divided into two tubes, with one maintained at 37 C and the other killed by incubation at 56 C for one hour. A portion of the live and dead bacteria were opsonized by the addition of equal volumes of autologous serum and incubated at 37 C for 30 minutes in a shaking water bath. Live and dead organisms (unopsonized) were included to ensure that all organisms received the same physical treatments. All suspensions were washed twice and centrifuged (1000 x g) at 4 C for 10 minutes before final resuspension. The final preparations were maintained at room temperature.

Chemiluminescence: Chemiluminescence (LECL) was measured in a liquid scintillation counter[m] calibrated with

[1]Shimadzu UV-260, Shimadzu Instruments LTD, Tokyo, Japan.
[m]Beckman LS7500, Beckman Instrument Co., Fullerton, CA.

a tritium standard just prior to use. For each agonist, luminol and PMN's (1 X 10^6) were added to a polyethylene minivial[n], mixed by inversion, placed into a 20 ml polyethylene counting vial and lowered into the counting chamber. After three baseline counts, the agonist was added, and the vial was inverted five times and returned to the counting chamber for 15 minutes. The end volume in all vials was 1.0 ml. Each reaction vial was counted at 0.1 minute intervals for 15 minutes. To ensure that the time after isolation of cells did not influence experimental results, the sequence of agonists was changed daily. Adjustment of the instrument's sigma error from 2% to 0.01% made it possible to perform all assays utilizing a 70 uM concentration of luminol, which enhanced the detection sensitivity of emitted LECL without exceeding the instrument's counting linearity. All counts were obtained with the instrument in an in-coincidence mode. The instrument counted [in counts per minute (CPM)] and printed five data points per minute (of elapsed time).

For analysis of data, the five data points were averaged and log-transformed. Peak LECL, initial response time, and time to peak response were recorded. Peak LECL was defined as the highest CPM recorded during the 15 minute assay time period. Initial response time (IRT) was defined as the time (in minutes) when the average of 5 counts more

[n]Beckman Instrument Co., Fullerton, CA.

than doubled the average background counts. In most instances, the IRT count far exceeded the background count. The time to peak response (TTPR) was defined as the elapsed time (in minutes) of the highest (peak) count over the 15 minutes of recorded LECL. Because the peak responses to some agonists (OZ, FMLP, ZAS, bacteria) were prolonged, and did not occur within the 15 minute period of recorded LECL, data analyses for comparative studies were also based on the slope of LECL responses over time. The slope responses were calculated by using the IRT count (log transformed) as the first data point and the next four data points. Data analysis for differences in neutrophil responses between groups of agonists was by means of a computer based statistical program[m] for one way analysis of variance (ANOVA, OWA), assuming $P \leq 0.05$ as significant. Where there was significance in the ANOVA design, comparisons of means was by the least-significant-difference (LSD) test[o].

Specificity of LECL: The specificity of the LECL response to each agonist was investigated by the inclusion of SOD in the reaction vials, thereby defining the contribution of superoxide anion to the generation of LECL. The percentage of SOD inhibition for each agonist was compared by means of one-way analysis of variance (OWA).

Superoxide Assay: The production of superoxide anion was measured by the superoxide dismutase-inhibitable

[o]SAS Insitute Inc., Cary, NC.

reduction of ferricytochrome C, as previously described.[53,54] Briefly, each reaction tube contained 2 X 10^6 neutrophils, ferricytochrome C, and each agonist with or without SOD (total volume 1.0 ml). Two sets of paired tubes were used for each agonist tested and for the unstimulated control with the SOD-containing tubes serving as the reference blanks for the non-SOD-containing tubes. One tube contained only ferricytochrome C in HBSS. All tubes were placed in a shaking water bath[j] for 30 minutes at 37 C. After incubation, the reaction was stopped by the addition of SOD to the tubes without SOD, HBSS was added to each bringing the final volume to 2.0 ml, and the tubes were centrifuged at 1000 X g for 20 minutes. After centrifugation 1.5 ml of the supernatant from each tube was pipetted into disposable semi-micro cuvettes[n] and absorbances were read in a double-beam spectrophotometer[l], initially blanked using HBSS in the sample and reference positions. With HBSS as the reference, the ferricytochrome control was scanned (between 540 and 560 nm) with the peak absorbance at 550 nm recorded. The supernatant was reduced with sodium dithionate and rescanned. The peak optical density was consistently less than 0.750 assuring linearity of results.[54] All samples were read sequentially in a similar manner. The absorbance, after adding dithionate, was indicative of the total amount of ferricytochrome C

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[p]Cole Parmer Instrument Co., Chicago, IL.

available for reduction. The net nanomoles of superoxide produced were obtained by subtracting the nM O_2- produced by unstimulated controls from those produced by each agonist. The values for each set of two tubes were averaged. The mM extinction coefficient for a 1 cm light path Fe^{2+} Cytochrome C (reduced)-ferricytochrome C (oxidized) was assumed to be 21.1.[54] Values were recorded as nM O_2-/2 X 10^6 cells/30 minutes. Analysis of data for significance ($P \leq 0.05$) between treatment groups used a OWA with LSD comparison of means.

Effect of the Age of the Calf On Total and Differential WBC Counts: The effect of age on the total and differential neutrophil counts from each calf was determined by means of a general linear model (GLM) one way ANOVA[m] with LSD comparisons of significant ($P \leq 0.05$) means. For these studies, the seven day period starting at the time of purchase was designated as week one (the initial week of acclimation). Subsequent weeks were designated as week two and week three, respectively. The study was limited to a three-week period.

Effect of Blood Handling: The effect of maintaining collected blood and isolated cells at room temperature or in ice was evaluated by paired T (PRT) tests[m] for both assays (Tables 1.1 & 1.2). Graphic depiction of these experiments is shown in Figures 1.1 and 1.2. These tables and graphs will follow "determination of agonist levels"

(Materials and Methods). Room temperature significantly enhanced LECL, whereas iced cells produced a more significant superoxide response. Since the production of superoxide anion was enhanced and since its production has been postulated to have a major role in the generation of LECL all blood samples and isolated cells were routinely maintained in ice. The maintenance of blood samples and isolated cells in ice also reduced the background counts (LECL) and the superoxide production of unstimulated control cells to consistently low levels (data not shown).

Determination of Agonist Levels: In dose response studies of LECL and superoxide production agonist concentrations which produced maximal stimulation of human neutrophil oxidative metabolism[53,54] were used. The effects of different agonist concentrations were analyzed by use of OWA with comparison of means using the LSD test. Two concentrations of agonists or treatments were analyzed by PRT tests. To determine the role of complement factors, immunoglobulins and host recognition factors as opsonins for zymosan, heated, unheated, autologous and homologous sera were compared by paired T tests in each assay. Since the use of heated serum for opsonization of zymosan markedly diminished neutrophil LECL and superoxide responses, over unheated serum, the amounts (percentages) of inhibition in both assays were compared by a GLM one-way ANOVA with LSD comparison of means. For the best neutrophil responses,

zymosan (10 mg/ml) was opsonized with unheated, autologous serum. This quantity of zymosan gave an approximate ratio of 25 particles per neutrophil.[54] To ensure consistency of stimulation, dose responses were repeated for newly prepared batches of soluble agonists.

For assessing LECL, latex particles, phorbol ester (PMA), calcium ionophore (CI) and opsonized zymosan (OZ) were used. Because latex particles were difficult to remove from the supernatants of superoxide assays and adversely affected absorbance, OZ was the only particulate agonist used in the superoxide assays, in addition to the soluble agonists PMA and CI. The factors which were investigated in determining agonist concentrations for LECL are summarized in Table 1.1 and graphically in Figure 1.1, and those for superoxide production are summarized in Table 2-2 and graphically in Figure 1.2. The concentrations selected were 50 ul of latex particles (LECL only), 200 ng of PMA, 10^{-5} M CI, and 50 ul of opsonized zymosan. To ensure that cells were exposed to identical amounts of particulates, 100 ul of OZ were used in the superoxide assay (containing 2 X 10^{6} cells). Identical concentrations of soluble agonists were used in both LECL and superoxide assays. Analysis of data for LECL generation and superoxide production was by OWA with LSD comparison of means where significant ($P \leq 0.05$).

Effect of Individual Calf Variability and Age On LECL and Superoxide Production: To evaluate the effects of the

Table 1.1. Selection of Agonist Concentrations For Use in Studies of Luminol-Enhanced Chemiluminescence by Bovine Neutrophils.

Agonist or treatment	N	Method	P Value if Sign.	Concentration or treatment selected
Room temperature vs iced cells	5	PRT	0.0296	Iced Cells#
Latex (25, 50, 75, 100 ul)	5	OWA	N.S.	50 ul
Phorbol ester (PMA) (1000, 500, 200, 100, 50 ng)	5	OWA	N.S.	100 ng
Calcium ionophore (CI) (10^{-5} M, 10^{-6} M, 10^{-7} M)	5	OWA,LSD	0.0001	10^{-5} M
OZU vs OZH*	5	PRT	0.0028	OZU
AOZ vs HOZ**	5	PRT	0.0008	AOZ

OWA=One Way ANOVA; LSD=least-significant-difference; PRT=paired T test; NS=not significant; If significant, P>T given; OZ=opsonized zymosan; *Zymosan opsonized with heated (OZH) or unheated (OZU) serum;**Zymosan opsonized with autologous(AOZ) or homologous (HOZ) serum; N=number of experiments; Analyses shown graphically in Figure 1.1; #See text for explanation.

Figure 1.1. Graphic Representation of Experiments Performed in the Selection of Treatments and Agonist Concentrations for Use in Studies of Luminol-Enhanced Chemiluminescence (Table 1.1) by Bovine Neutrophils.

Different bar patterns represent the means for each comparison group. Two agonists or treatments were analyzed by paired "T" tests. Three or more agonist concentrations were analyzed by one-way ANOVA. RT=neutrophils kept at room temperature; Iced=neutrophils kept in crushed Ice. OZU=zymosan opsonized with unheated autologous serum; OZH=zymosan opsonized with heated autologous serum; AOZ=zymosan opsonized with autologous serum; HOZ=zymosan opsonized with homologous serum; Paired T test analyses of room temperature versus iced cells and AOZ versus HOZ were performed on untransformed peak counts, and were log transformed for graphic purposes only. Horizontal bracket with a centered asterisk (*) enclose groups that were significantly different; horizontal bar with a subtitle NS enclose groups with no significant differences.

Luminol-Enhanced Chemiluminescence Log10 (CPM)
0
1
2
3
4
5
6
7
*
NS
NS
*
*
*
RT
Iced
25
50
75
100
1000
500
200
100
50
10
1
10-3
10-6
10-9
OZU
OZH
AOZ
HOZ
Latex (µl used)
Phorbol Ester (ng/ml)
Calcium Ionophore (M)
Opsonized Zymosan (10mg/ml)

Table 1.2. Selection of Agonist Concentrations for Use in Studies of Superoxide Production by Bovine Neutrophils.

Agonist or Treatment	N	Method	P Value if Sign.	Concentration or treatment selected
Room Temperature vs Iced Cells	6	PRT	0.0001	Iced Cells
Phorbol ester (PMA) (1000 ng, 100 ng, 10 ng)	6	OWA	N.S.	100 ng
Calcium ionophore (CI) (10^{-5} M, 10^{-6} M)	6	PRT	0.0264	10^{-5} M
Z10 vs Z20 (50 ul)	2	PRT	N.S.	N/A
Z10 vs OZ10 (50 ul)	8	PRT	0.0001	OZ 10
AOZ vs HOZ**	8	PRT	0.0017	AOZ
OZU vs OZH*	4	PRT	0.0296	OZU
50 ul OZ10 vs 100 ul OZ10	3	PRT	N.S.	50 ul
OZ5 vs OZ10 vs OZ20 (50 ul)	4	OWA	N.S.	OZ 10

OWA=one-way ANOVA; PRT=paired T test; NS=not significant; Z=zymosan; Z10=zymosan, 10 mg/ml; Z20=zymosan, 20 mg/ml; OZ10=opsonized zymosan, 10 mg/ml; OZ5=opsonized zymosan 5 mg/ml; OZ20=opsonized zymosan 20 mg/ml; *zymosan opsonized with heated (OZH) or unheated heated (OZU) serum; **zymosan opsonized with homologous (HOZ) or autologous (AOZ) serum; N=number of experiments; N/A=not applicable. Analyses shown graphically in Figure 1.2.

Figure 1.2. Graphic Representation of Experiments Performed in the Selection of Treatments and Agonist Concentrations for Use in Studies of Superoxide Production (Table 1.2) by Bovine Neutrophils.

Different bar patterns represent the means for each comparison group. Two agonists or treatments were analyzed by paired "T" tests; three or more agonist concentrations were analyzed by one-way ANOVA; Z10-zymosan (10 mg/ml); Z20-zymosan (20 mg/ml); OZ10-opsonized zymosan (10 mg/ml); 50 ul-fifty microliters of opsonized zymosan (10 mg/ml); 100 ul-one hundred microliters of opsonized zymosan (10 mg/ml); OZ5-opsonized zymosan (5 mg/ml); OZ20-opsonized zymosan (20 mg/ml). All other abbreviations identical to figure 1.1. Horizontal brackets with a centered asterisk (*) enclose treatment groups that were significantly different; horizontal brackets with the subtitle NS enclose groups with no significant differences.

NM Superoxide/2x10^6 Neutrophils/30 min.
40
30
20
10
0
Iced
RT
100
100
10
10
10-8
10-8
10
10
20
20
10
10
40
40
20
20
50ul
50ul
25
25
20
20
NS
NS
NS
NS
*
*
*
*
*
Phorbol Ester (Ng/ml)
Calcium Ionophore (M)
Zymosan and Opsonized Zymosan

variability among calves and the effect of age on neutrophil responses, randomized completely blocked ANOVAs, using calves as a block and age as a treatment effect, were performed for each agonist and for each assay. Analyses for significant differences of the treatment effects (age), where significant ($P \leq 0.05$), was by LSD comparison of means. Since the actual studies were initiated during the second week after arrival, these data analyses were for weeks two, three, and four after arrival, respectively. The calves at those times were approximately 2.5-3, 3.5-4, and 4.5-5 weeks of age respectively.

Comparisons to *Pasteurella haemolytica*: A series of experiments were performed in which the LECL peak and slope responses and superoxide production of neutrophils stimulated by agonists were compared to the responses stimulated by *Pasteurella haemolytica* (PH). In these comparative studies, live (PHL), dead (PHD), live-opsonized (PHLO), and dead-opsonized (PHDO) bacteria were used. The possible effects of formylated oligopeptides (FMLP) and complement factors [C5a (ZAS)] on the oxidative metabolism of bovine neutrophils were evaluated by their inclusion in these protocols. The quantities of bacterial suspensions used, 50 ul for LECL and 100 ul for superoxide assays gave an approximate ratio of 25 bacteria per neutrophil[134] and was nearly identical to the particle to cell ratio obtained with OZ.[54] The concentration of FMLP (10^{-5} M) was that

indicated to induce maximal oxidative response in other species.[54] The level of ZAS used has been previously defined[136] for bovine neutrophils. Analysis of data for these studies was by OWA with LSD comparisons of means, where significant ($P \leq 0.05$).

RESULTS:

Neutrophil isolation techniques consistently yielded >95 % neutrophils of >95% viability as determined by differential counts and trypan blue dye exclusion tests. Contaminating cells were principally lymphocytes with few platelets and red blood cells. Because our protocols were limited to calves less than 5 weeks of age, there were no problems with eosinophil contamination. There were significant decreases in total WBC counts and in the percentage of neutrophils between week one and week two, but there were no significant differences between week two and week three. (Table 1.3)

The heating (56 C for one hour) of autologous serum prior to use in the opsonization of zymosan particles, compared to the use of unheated serum, produced significant decreases in LECL and superoxide production responses (Tables 1.1 and 1.2, Figures 1.1 and 1.2). The percentage inhibition of LECL produced by the destruction of complement factors (heating of serum) was significantly greater than the percentage inhibition of superoxide production (Table 1.4).

Table 1.3. Effect of Weeks in the Study on Total White Blood Cell and Percent Neutrophil Counts[#]

	Total White Blood Cells	Percent Neutrophils
Week one	9.23 ± 0.96*	48.68 ± 2.02*
Week two	7.25 ± 0.64 NS)*	36.19 ± 3.61 NS)*
Week three	6.33 ± 0.36	33.91 ± 3.28

[#]analysis by General Linear Model (GLM) ANOVA with LSD comparison of means; Data reported in Mean ± Standard error of the mean; Total white blood cells=count X 10^3; *=groups differ significantly; NS)*=paired groups not significantly different

Table 1.4. Luminol-enhanced Chemiluminescence and Superoxide Production: Inhibition of the Responses to Opsonized Zymosan by the Use of Heated Serum for Opsonization of Zymosan*

Protocol	Percent inhibition (+/- SEM)
Superoxide	49.70 ± 10.12
LECL	**96.35 ± 0.61

*Analysis by General Linear Model ANOVA;**Significant at $P \leq 0.05$.

Table 1.3. Effect of Weeks in the Study on Total White Blood Cell and Percent Neutrophil Counts[#]

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[#]analysis by General Linear Model (GLM) ANOVA with LSD comparison of means; Data reported in Mean ± Standard error of the mean; Total white blood cells=count X 10^3; *=groups differ significantly; NS)*=paired groups not significantly different

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LECL	**96.35 ± 0.61

*Analysis by General Linear Model ANOVA;**Significant at $P \leq 0.05$.

Latex particles stimulated the highest LECL responses in all instances, and these responses were often an indicator of the maximal neutrophil response for each day's experiments. The LECL peak responses to latex, PMA, CI, and OZ were all significantly different from each other. The times of initial responses to latex particles were consistently shortest, those to PMA and CI statistically similar, and those to OZ the longest with all three treatment groups differing significantly. The time to peak response was shortest when neutrophils were exposed to PMA, but the response time was not significantly different from neutrophils exposed to latex. The peak response times for latex and PMA were significantly different from the responses to CI and to OZ. The responses to CI and OZ were also significantly different (Table 1.5, Figure 1.3).

Although the inclusion of superoxide dismutase (SOD) diminished the LECL peak responses of neutrophils to soluble agonists (PMA and CI) more than for particulate agonists (latex and OZ), there were no significant differences in the percentages of LECL-inhibition produced by SOD. (Table 1.6).

Phorbol ester consistently induced the maximal production of superoxide anion with the responses differing significantly from those to calcium ionophore and opsonized zymosan, which were not significantly different (Table 1.7).

Table 1.5. Bovine Neutrophil Oxidative Responses: Luminol-enhanced Chemiluminescence: Peak Response, Initial and Peak Response Times#

Agonist	Peak Response Log_{10}(CPM)	Initial response Time (Minutes)	Peak Response Time (Minutes)
Latex	5.49 ± 0.14*	1.50 ± 0.1*	9.41 ± 0.44 NS)*
PMA	4.14 ± 0.05*	2.71 ± 0.13 NS)*	7.88 ± 0.32
CI	3.80 ± 0.14*	3.03 ± 0.15	11.38 ± 0.61*
OZ	2.97 ± 0.07*	4.71 ± 0.25*	14.94 ± 0.04*

Data in mean +/- SEM; #Analysis by One-way ANOVA and Least-Significant-difference; PMA=phorbol ester; CI=calcium ionophore; OZ=opsonized zymosan; *all groups significantly different; NS)*=pair did not differ significantly; N=35.

Table 1.6. Luminol-enhanced Chemiluminescence: Comparison of Percentage Inhibition of Neutrophil Responses to Agonists by Superoxide Dismutase*

Agonist	Percentage inhibition (+/- SEM)
Latex	**55.23 ± 9.14
Phorbol ester (PMA)	**77.20 ± 7.42
Calcium ionophore (CI)	**62.31 ± 11.59
Opsonized zymosan (OZ)	**55.14 ± 5.88

*Analysis by One-way ANOVA; N=5; **=Not significant

Figure 1.3. Bovine Neutrophil Luminol-enhanced Chemiluminescence (LECL): Comparison of the Peak Responses, Initial Response Times, and Peak Response Times Induced by Soluble and Particulate Agonists.

Analyses by one-way ANOVA (OWA) with comparisons of significant ($P \leq 0.05$) means by the least-significant-difference (LSD) test. Each bar represents the mean of each treatment group, and the responses induced by each agonist. *=Significant differences between treatment groups. The horizontal bracket and subtitle NS represents treatments that were not significantly different between enclosed groups. N=number of experiments.

Luminol-enhanced Chemiluminescence Log 10(CPM)
Latex
PMA
CI
OZ
N=35
Initial Response Time (minutes)
SN
Peak Response Time (minutes)
SN

Table 1.7. Bovine Neutrophil Oxidative Responses: Superoxide Production in Response to Soluble and Particulate Agonists.

PMA: 25.86 ± 0.95*	CI: 8.17 ± 0.82**	OZ: 6.39 ± 0.52**

Data in mean ± Standard error of the mean; Reported in nM O_2^-/2 X 10^6 cells/30 min; PMA=phorbol ester, CI=calcium ionophore; OZ=opsonized zymosan; *significantly different from other treatment groups. **groups did not differ significantly; Analysis via OWA and LSD; N=35.

Table 1.8. Effect of Calf and Weeks in the Studies (Age) on Luminol-enhanced Chemiluminescence and Superoxide Production Responses to Particulate and Soluble Agonists.

Stimulus	Protocol	N	Calf effect	Age effect
Latex	LECL	7	0.0014	NS
PMA	O_2^-	7	NS	NS
PMA	LECL	5	NS	NS
CI	O_2^-	5	NS	NS
CI	LECL	4	0.0106	0.0238**
OZ	O_2^-	5	0.001	NS
OZ	LECL	8	0.001	0.0252**

*Analysis by randomized complete block ANOVA; PMA=Phorbol ester; CI=calcium ionophore; OZ=opsonized zymosan; LECL=luminol enhanced chemiluminescence; O_2^-=superoxide production; If significant P>T value given; NS=not significant; Week two=calves 2.5-3 weeks old; Week three=calves 3.5-4 weeks old; Week four=calves 4.5-5 weeks old; **significantly different between week three and week four.

Randomized complete block analyses showed calf effects in the LECL peak responses to latex, CI, and OZ, but not in the response to PMA. There were significantly diminished responses between week 3 and week 4 with CI and OZ. For superoxide production only OZ had a significant calf effect but there were no significant effect of age for any agonist. (Table 1.8)

Comparisons of the LECL peak responses of neutrophils to agonists and to the *Pasteurella* organism, FMLP, and ZAS are depicted in Figure 1.4. The magnitude of neutrophil peak responses to latex, PMA, and CI were all significantly greater than to any of the preparations of *Pasteurella* organisms. Although differing significantly from the neutrophil responses to latex, PMA, and CI, the responses to live opsonized *Pasteurella* organisms (PHLO) were statistically equal to the responses to OZ. The opsonization of live *Pasteurella* organisms significantly enhanced the neutrophil LECL response over PHL, PHDO, and PHD. While the cell responses to OZ did not differ significantly from those to live *Pasteurella* (PHL) or to dead-opsonized *Pasteurella* (PHDO), they were significantly different from FMLP, ZAS, and dead *Pasteurella* organisms (PHD).

Analysis of neutrophil slope response data (Figure 1.5) from the same series of protocols revealed significant

Figure 1.4. Bovine Neutrophil LECL: Comparisons of the Peak Responses Induced by Soluble and Particulate Agonists and Those Induced by *Pasteurella haemolytica*, Formyl-Methionyl-Leucyl-Phenylalanine (FMLP) and Zymosan-Activated Serum (ZAS).

Analyses by OWA and LSD. LTX=latex particles; PMA=phorbol ester; CI=calcium ionophore; PHLO=live opsonized *Pasteurella*; OZ=opsonized zymosan; PHL=live *Pasteurella*; PHDO=dead opsonized *Pasteurella*; PHD=dead *Pasteurella*; each bar represents the mean of each treatment group with a standard error bar. Horizontal brackets with a centered asterisk (*) enclose groups that are significantly different. Bracket with a subtitle NS enclose groups that are not significantly different. N=number of experiments.

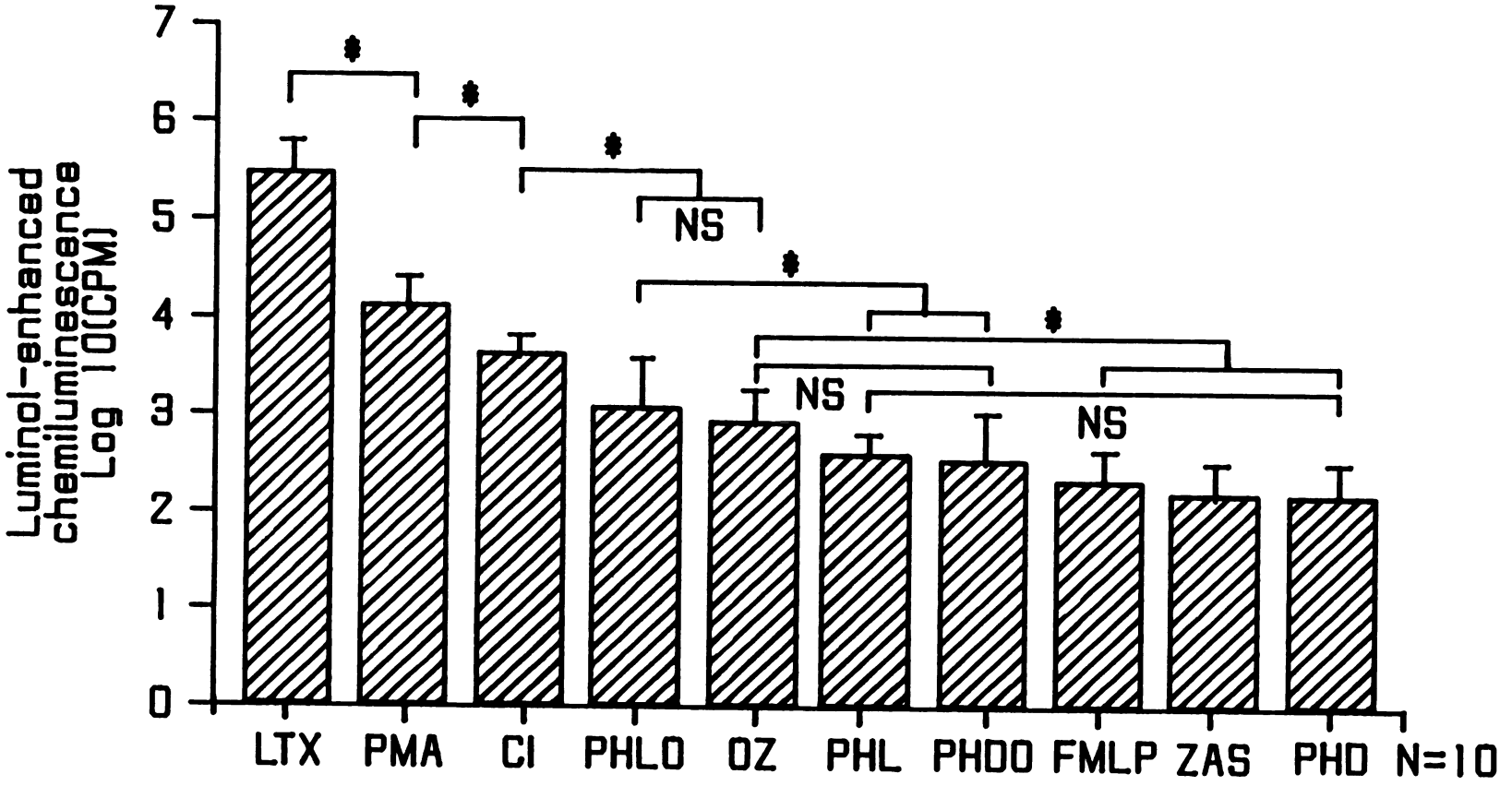
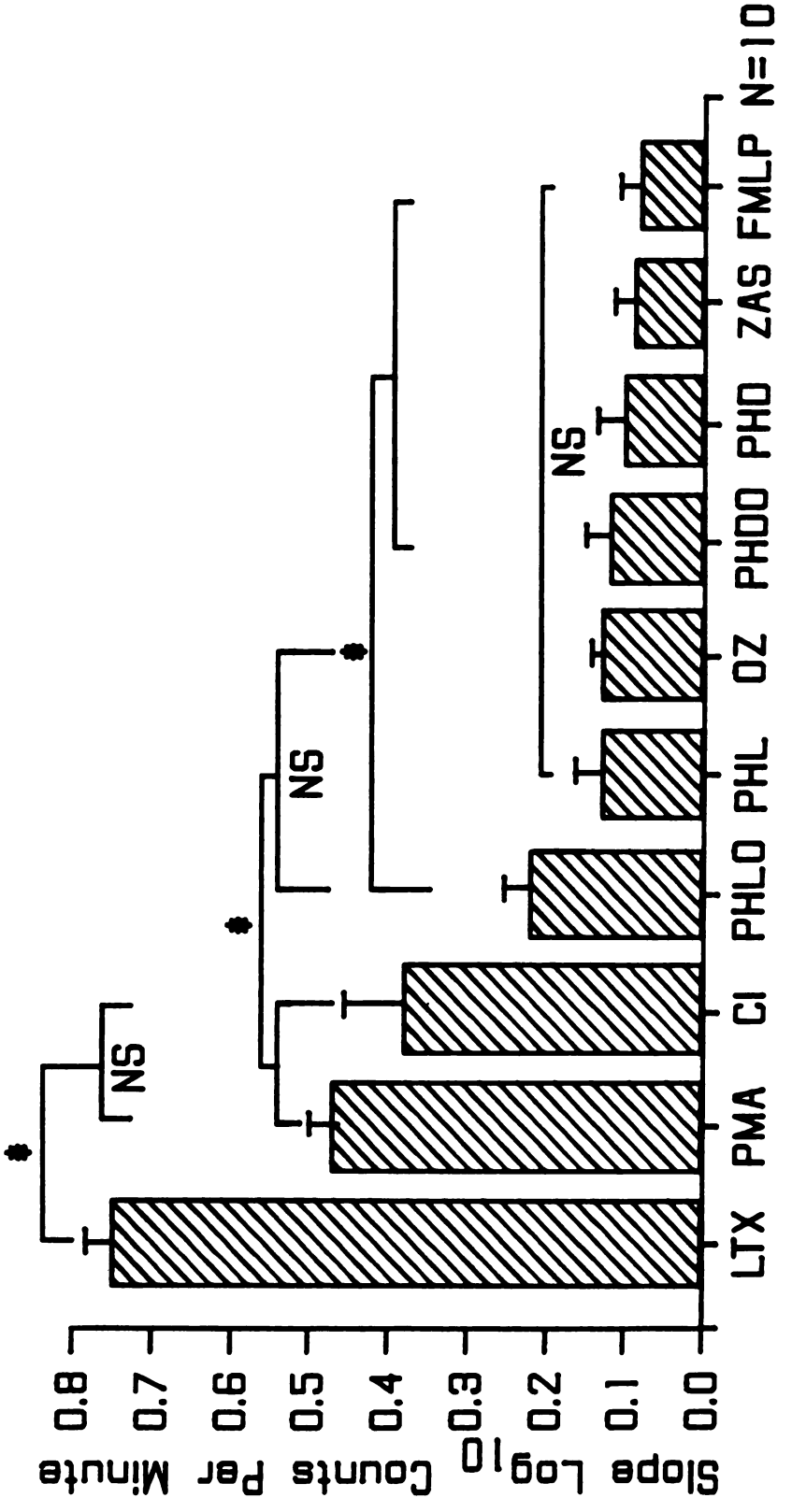

Luminol-enhanced chemiluminescence Log 10(CPM)
7
6
5
4
3
2
1
0
*
*
*
NS
*
*
NS
NS
LTX
PMA
CI
PHLO
OZ
PHL
PHDO
FMLP
ZAS
PHD
N=10

Figure 1.5. Bovine Neutrophil LECL: Comparisons of the Slope Responses Induced by Soluble and Particulate Agonists and Those Induced by *Pasteurella haemolytica*, FMLP, and ZAS.

Analyses by OWA and LSD. Abbreviations of agonist groups identical to figure 1.2. Each bar represents the mean of each treatment group with a standard error bar. Asterisk (*) centered over a horizontal bracket encloses groups which differ significantly. Bracket with a subtitle NS enclose groups that are not significantly different. N=number of experiments.


Slope Log10 Counts Per Minute
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
NS
NS
NS
*
*
*
LTX
PMA
CI
PHLO
PHL
OZ
PHOO
PHO
ZAS
FMLP
N=10

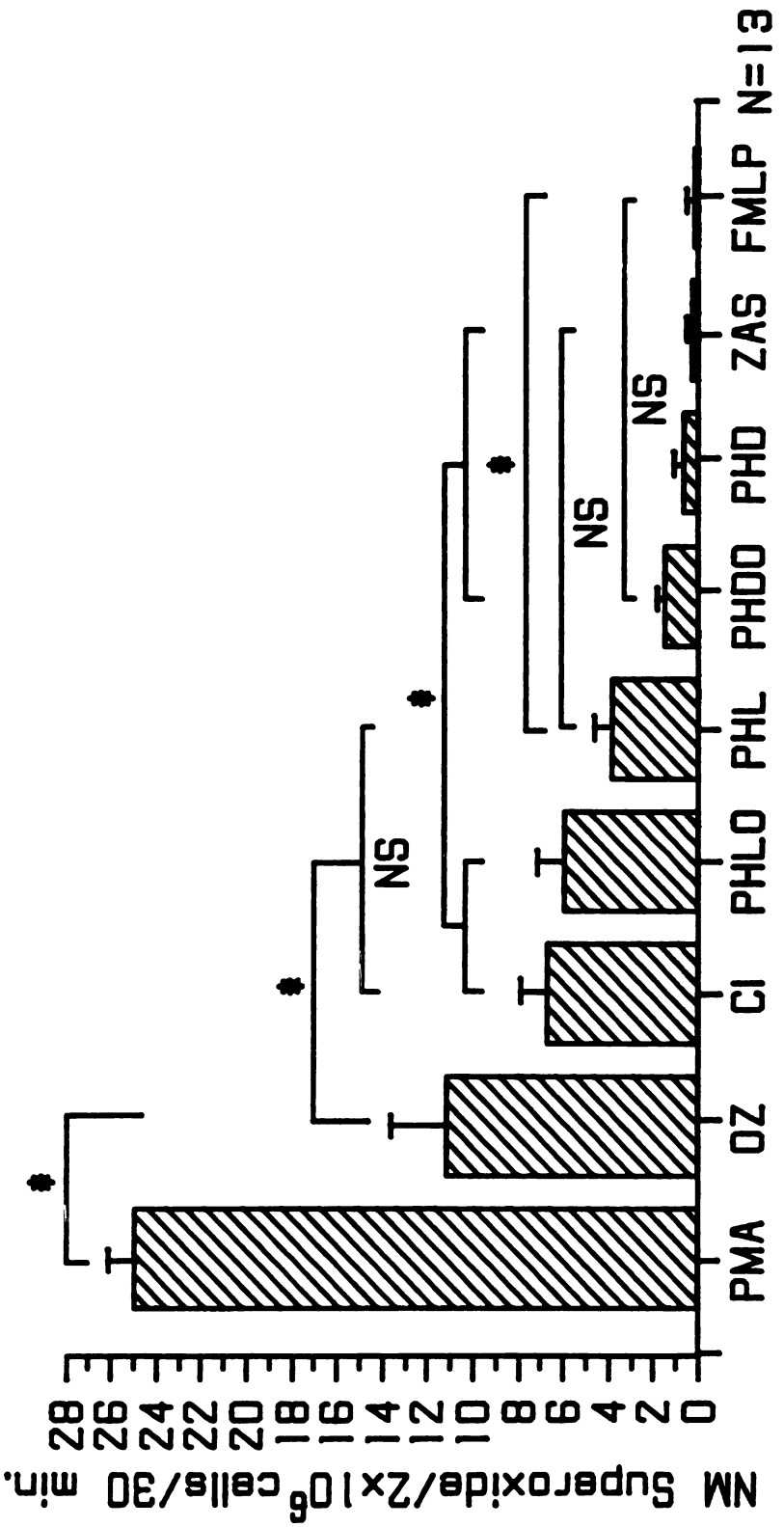
differences between the responses to latex, the twosome of PMA and CI, and the threesome of PHLO, PHL, and OZ. The latter two groups did not differ significantly within each respective group. The slope responses to PHLO differed significantly from those to PHDO, PHD, ZAS and FMLP.

Superoxide production is depicted in Figure 1.6. The bovine neutrophil superoxide production responses to PMA and OZ were significantly different from each other and from the responses to CI, PHLO, and PHL. The responses to CI and PHLO were significantly different from those to PHDO, PHD, and ZAS.

DISCUSSION: Neutrophils have come under intense biological scrutiny in the past decade, with the publication of over 25,000 scientific articles in the last five years alone. This interest has been not only in the definition of neutrophil cellular biology, but in the role of neutrophils and neutrophil products in disease. A number of diseases have been identified as neutrophil-mediated through neutrophil depletion studies in experimental animals.[128,129,182] Recently, Slocombe et.al[129] performed neutrophil depletion studies in neonatal calves and defined the role of the neutrophil in the pathogenesis of lung injury in experimentally-induced pneumonic pasteurellosis.[129] However, the definition of the neutrophil product(s) responsible for the characteristic gross and microscopic lesions, has been hampered by the complexity of

Figure 1.6. Bovine Neutrophil Superoxide Production: Comparison of Responses Induced by Soluble and Particulate Agonists and Those Induced by *Pasteurella haemolytica*, FMLP, and ZAS.

Analyses by OWA and LSD. Abbreviations of agonist groups identical to figures 1.2 and 1.3. Each bar represents the mean of each treatment group with a standard error bar. Horizontal brackets with a centered asterisk (*) enclose groups that are significantly different. Bracket with a subtitle NS enclose groups that are not significantly different. N=number of experiments.


NM Superoxide/2x10⁶ cells/30 min.
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
PMA
OZ
CI
PHLO
PHL
PHDO
PHD
ZAS
FMLP
N=13
NS
NS
NS
*
*
*
*

interrelated *in vivo* pathways, necessitating *in vitro* studies for delineation.

In the comparative studies of neutrophil responses to defined concentrations of agonists versus the responses to *Pasteurella* organisms, the magnitudes of neutrophil LECL (peak and slope) responses to latex, PMA, and CI were significantly greater than responses to OZ and live opsonized *Pasteurella* organisms (PHLO). For superoxide production the responses to PMA and OZ were significantly higher than to PHLO and CI. Whether the differences in the magnitudes of the LECL and superoxide responses are of biological importance is difficult to assess since the highest responses were to non-biological entities.

What is of major importance are the similarities of the neutrophil responses to PHLO and OZ, since both particles are opsonized via the alternate pathway and because the particle to cell ratios (25:1) were approximately equal.[54,133] The differences in LECL and superoxide production responses stimulated by OZ and PHLO suggest: 1) that differences in the mechanisms of induction of LECL and superoxide production by similar particulate agonists must exist, or 2) that difficulties exist in the attempted correlation of LECL and superoxide production data. Since the generation of LECL has been attributed to the production of reactive oxygen species such as singlet oxygen, hydroxyl ion, superoxide anion, hydrogen peroxide and hypohalous

ion, it is conceivable that the attempt to correlate the effects of five luminol-reactive entities with the production of one (superoxide) would be difficult even when induced by similar or identical agonists. It is also conceivable that a single agonist could induce the production of a decidedly different spectrum of these compounds due to the influence of other, but presently undefined, factors.

The similarities in the neutrophil responses to PHLO and OZ suggests that the mechanisms of neutrophil transduction by each are similar, although OZ induces the production of more superoxide. Since studies of Chang et. al.[133] have shown that the ingestion of *Pasteurella haemolytica* by bovine neutrophils leads to a rapid diminution of LECL, it is possible that the pathogens may have altered the production of superoxide, thereby explaining the differences between OZ and PHLO in superoxide production.

The bovine neutrophil responses to the different treatments of *Pasteurella* were incremental, although not necessarily significantly different, with PHLO inducing the highest responses, followed in order by PHL, PHDO, and PHD. These data suggest: 1) that there is a factor produced by live organisms that enhances their uptake by neutrophils, 2) that the effect of this factor is enhanced by opsonization, 3) that the uptake of dead bacteria by neutrophils is also facilitated by opsonins, and 4) that the phagocytosis

of opsonized particulates, nonpathogenic (OZ, PHDO) or pathogenic (PHLO) induces a substantial release of reactive oxygen species (LECL), including superoxide anion. Because it is not possible to accurately quantitate the release of reactive oxygen species in tissues, the postulation that these *in vitro* events may be related to the pulmonary injury seen in pneumonic pasteurellosis is hypothetical. While the factor(s) which induce these responses, other than opsonins, is/are presently undefined, it is apparently not a formylated oligopeptide, such as FMLP, since bovine neutrophils do not have FMLP receptors[99], and since the oxidative metabolism of bovine neutrophils induced by exposure to FMLP was minimal. The role of complement factors in these responses was minimized because exogenous complement (ZAS) failed to induce significant oxidative metabolism. Although complement activation can occur *in vitro* by means of the release of specific granule components[185], there is a requirement for the presence of serum. Serum-free media was used in all of these experiments.

The pathogenesis of neutrophil-mediated injury has been postulated to be the secretion and/or release of toxic reactive oxygen species formed as a consequence of hexose monophosphate (HMP) shunt activation, the secretion and/or release of enzymes from cytoplasmic granules, and the production of arachidonic acid metabolites.[1-3] The series of

events culminating in neutrophil activation is complex. A series of soluble and particulate agonists have been utilized in an attempt to delineate these processes. The interaction of surface membrane receptors with diffusible or particulate agents and membrane perturbation facilitating ionic transport may directly or indirectly induce intracellular enzyme systems, and/or stimulate the release or production of second messengers. These may then be important in the generation of reactive oxygen species, cell motility, and secretion.[6] The complexity of transduction mechanisms is exemplified by the fact that some agonists may induce oxidative metabolism, others selective secretion, yet others motility or combinations of responses. There are phylogenetic differences in neutrophil metabolism, responses, and enzyme components, making interspecies comparisons difficult.[98,99] There have been no published studies which have explored the oxidative responses of bovine neutrophils to the spectrum of agonists used in these experiments.

Agonists were selected for use in these experiments because they stimulate neutrophil transduction by differing mechanisms. The cell response to latex particles has been reported to occur as a function of particle size and the surface potential properties of the particles.[186] In our experiments in which the LECL responses of 1 X 10^6

neutrophils to four concentrations (25, 50, 75, and 100 ul/ml) of 0.8 micrometer latex particles were compared, there were no significant differences in cell responses. Mechanisms of transduction may therefore involve the initial perturbation of surface glycoproteins and sialic acid residues, leading to ionic transport with secondary activation of membrane-bound enzyme systems and perhaps the formation of intracellular second messengers. The extreme rapidity of the response to latex, as compared to the particulate OZ, suggests that the initial cell responses were due to membrane disturbances with later responses due to particle phagocytosis.

The induction of the NADPH-oxidase complex, the "respiratory burst" enzyme, has been attributed to the intracellular activation of protein kinase C (PKC) with translocation of PKC to the plasma membrane.[21] Tumor promoting substances such as phorbol ester (PMA) have been shown to directly activate PKC, bypassing the cascade of intracellular events normally involved in its activation.[19,20] The activation of PKC, although reported to be calcium-dependent, occurs at "vanishingly low" Ca^{2+} concentrations (10^{-8}-10^{-10}M).[20,21] In response to micromolar concentrations of calcium, PKC and a Ca^{2+}-requiring thiol proteinase (calpain) become associated with the plasma membrane. Calpain activation converts PKC into a proteolytically modified calcium and phospholipid-independent form

which is released into the cytosol where it has access to other, but presently undefined, protein substrates.[21,25-27]

The use of the compound A23187, also termed calcium ionophore (CI), facilitates the transport of the divalent cation Ca^{2+} into the cytosol where it has a major role as a second messenger.[4,6] Increases of intracellular Ca^{2+} may activate numerous calcium-dependent enzymes, including PKC, phospholipase C, phospholipase A_2, calmodulin, and calpain, all of which have roles in the mechanisms of neutrophil transduction. Cellular activation by the combination of CI and PMA, each at lower than "usual" concentrations for neutrophil effects, has been shown to mimic "normal" intracellular events.[24]

Particulate agonists, such as opsonized zymosan (OZ) and perhaps *Pasteurella haemolytica*, may activate the phosphoinositide cascade pathways, but stimulate the release of superoxide by a mechanism distinct from PMA, in that the stimulus is phospholipase A_2-mediated, calcium-dependent, and independent of PKC.[50] The release of arachidonic acid is also calcium-mediated in OZ-stimulated cells but not in PMA treated cells.[51] While the definitive mechanisms of OZ stimulation have not been defined, it has been suggested that the response may partially involve receptor-mediated events through interaction with surface C5a or Fc receptors.[50] Since the stores of these

receptors have been localized in the membranes of the specific granules, the addition of new surface membrane (and receptors) has been demonstrated upon the fusion of specific granules with the cell surface.[11-14] This process may also insert a cytochrome b component[32,34], which resides within the membranes of specific granules, into the membrane-bound NADPH-oxidase complex thereby completing activation and facilitating oxidative metabolism.

Studies of neutrophil oxidative metabolism have centered upon measures quantitating "respiratory burst" activity including chemiluminescence (CL) and the production of superoxide anion. The generation of CL, by neutrophils has been attributed to the production of compounds such as superoxide anion, hydrogen peroxide, singlet oxygen, hydroxyl ion, and hypohalous ions.[1,53-57] The use of luminol, a cyclic hydrazide which is oxidized to an electronically excited intermediate state (aminophthalate ion) which emits light photons upon relaxation to a ground state, has been used to augment "native" CL and increase sensitivity.[58-60] Studies of generated LECL have been utilized in the delineation of cellular oxidative metabolism and metabolic or acquired opsonophagocytic dysfunction.[60] The use of luminol also obviates the need for dark adaptation of vials and reagents and for the performance of the procedure under red (actinic) light.

Superoxide anion, produced by many enzyme systems, is formed either by the univalent reduction of oxygen or by the univalent oxidation of hydrogen peroxide.[1,5] In neutrophils, the induction of the NADPH-oxidase complex results in the formation of O_2- and H_2O_2 and leads to the activation and maintenance of the HMP shunt.[1,5,48] Superoxide and its metabolites, generated within phagolysosomes and released or secreted into the microenvironment has been postulated to have a primary role in the peroxidative attack on cell membranes, leading to cell injury or death.[5,45,46]

Both LECL and superoxide production protocols were characterized by extreme day-to-day variability in neutrophil responses. Although protocols for LECL and superoxide production used the same groups of isolated cells and the same concentrations of agonists, correlations in cell responses among agonists and assays were extremely difficult as addressed previously. These often diametrical responses were partially attributed to: 1) individual variability in neutrophil function, and 2) the quantities of superoxide anion induced by each agonist which contributed to the LECL response. Superoxide dismutase inhibited the neutrophil LECL responses to soluble agonists (PMA and CI) to a greater degree than to particulate agonists (latex and OZ), although

there were no significant differences in the percentages of inhibition between agonists (table 1.6).

The variability in cell responses described for LECL and superoxide production protocols was also apparent in the analyses for effects of age on neutrophil responses (Table 1.8). There were significant calf effects in neutrophil LECL responses to latex, CI, and OZ and significant age effects in the LECL responses to CI and OZ. These analyses confirmed the hypothesis of the variability in the LECL responses of individual calves. There was a significant decrease in the LECL responses to CI and OZ between 3.5-4 weeks of age and 4.5-5 weeks of age. For superoxide production, only OZ had a significant calf effect, but none of the agonists had an age effect. The reasons for the diminution in the LECL cell responses to CI at the ages studied are uncertain, but they could reflect alterations of Ca^{2+}-dependent enzymes or altered Ca^{2+} transport leading to decreased oxidative activity. The lack of similar alterations of CI-stimulated superoxide production suggests that this effect was not due to decreased superoxide production. Since neutrophil responses to OZ are mediated by a calcium-dependent enzyme, phospholipase A_2, and are also calcium-dependent[50], the alterations discussed above could also influence its responses. These findings suggest that individual variability and undefined effects of age in the responses of neutrophils to particulates could explain

increased susceptibility to bacteria, such as *Pasteurella haemolytica*, supporting the hypothesis of Hauser et. al.[127] that age-related variations in neutrophil function may influence the susceptibility to disease.

The response of neutrophils to particulates is partially dependent upon opsonization, as shown by the responses to *Pasteurella* organisms and opsonized zymosan in our experiments, with serum complement factors and immunoglobulins having major roles.[164] The use of heated serum for opsonization of particulates (OZ) produced a more significant degree of cell response inhibition in LECL than in production of superoxide (table 1.4), suggesting that complement fragments (and receptors) are more important for the generation of LECL and that immunoglobulin fragments (Fc receptors) are more important for superoxide production. In calves, the increased susceptibility could be principally due to lack of serum opsonic factors since neonatal calves are both hypogammaglobulinemic and hypocomplementemic at birth.[164] While immunoglobulins are passively acquired through ingestion and intestinal absorption, complement factors are not, and normal (adult) levels are not reached until approximately six months of age.[145] In calves, IgG1 is preferentially absorbed over IgG2 by an approximate ratio of 7:1.[141] The principal opsonic immunoglobulin in cattle serum remains a subject of debate with IgG1, IgG2, and IgM all purported to have the major

roles.[141-144] Although IgG1 and IgG2 fix complement by the classical pathway, only IgG2 fixes complement by the alternate pathway.[141,143] IgG2 also facilitates neutrophil phagocytosis.[143] If IgG2 is the principal opsonin, a selective IgG2 deficiency could explain the increased susceptibility of calves to gram-negative organisms.

Since a portion of the host defense against gram-negative organisms, such as *Pasteurella*, is dependent upon the alternate complement pathways, even adequate levels of immunoglobulins might not be protective. There have been few studies[147] quantitating the levels of classical and alternate pathway components with increasing age in calves. No one has attempted to correlate the quantities of classical and alternate pathway proteins with opsonophagocytic dysfunction. The use of adult and neonatal serum as opsonins for neonatal neutrophils, combined with the dependence of the classical and alternate pathways on Ca^{2+} and Mg^{2+}, respectively, could allow definition of the role of each component in host defense.

The data obtained in these comparative protocols will provide baseline information for further investigations into the role of neutrophil oxidative responses to the *Pasteurella* organism and/or its metabolic byproducts. Recently, several papers have been published which described the use of LECL-inhibition as a means of assaying the quantities of *P. haemolytica* cytotoxin through effects

on bovine neutrophils.[133-135] The extremes of LECL day-to-day variability, within individual animals, as well as between animals, casts doubt as to the validity and reproducibility of this assay. Although LECL was inhibited, there was no definitive proof that the neutrophils were actually killed. Other bacterial species, such as *Haemophilus somnus* and *Brucella abortus*, may produce low molecular weight adenine and guanine nucleotides which may inhibit the ingestion of radiolabeled staphylococci and diminish iodination.[115-120] If similar compounds are produced by *Pasteurella haemolytica* LECL might be inhibited as well. Since the induction of neutrophil oxidative responses to particulate entities[21,50] is mediated by different intracellular pathways than are responses to soluble[21] or receptor-mediated[15,17] agonists, perhaps the cytotoxin selectively blocks only these pathways, while others remain intact.

In the attempted definition of disease, *in vivo* studies are often complicated by the diversity of biological pathways, agonistic and antagonistic, which have major roles in the induction, prevention, exacerbation or modulation of tissue injury. In the attempts to define the pathogenesis of disease, particularly in man, animal models are extremely important. Future studies must of necessity involve the interpolation of *in vitro* results to *in vivo* events. The further definition of the role of

neutrophil products, and the attempted modulation of the factors responsible for injury, in pneumonic pasteurellosis may facilitate the understanding and possible treatment of fulminant gram-negative pneumonias in other species, including man, with obvious side benefits to the cattle industry.

In summary we have: 1) defined methods for the use of luminol-enhanced chemiluminescence and superoxide production in investigations of the oxidative metabolism of bovine neutrophils; 2) established concentrations of soluble and particulate agonists that maximally stimulate the oxidative metabolism of bovine neutrophils; 3) defined parameters of blood handling and agonist preparation that may influence the results of experiments; 4) determined that individual variability and age of calves may influence the responses to certain agonists; 5) determined that the responses of bovine neutrophils to *Pasteurella haemolytica* are highly dependent upon the presence of opsonins and/or live organisms and that opsonized live, live, or dead opsonized organisms stimulate the production of substantial amounts of reactive oxygen species, including superoxide anion, and 6) concluded that the production of these compounds may have a partial role in the lung injury which results from the interaction of neutrophils and *Pasteurella haemolytica*.

CHAPTER 2. THE EFFECTS OF ENDOTOXIN ON THE OXIDATIVE METABOLISM OF BOVINE NEUTROPHILS: COMPARATIVE EFFECTS OF *PASTEURELLA HAEMOLYTICA* AND *ESCHERICHIA COLI* ENDOTOXINS

INTRODUCTION: Bacterial lipopolysaccharide, also termed endotoxin, is derived from the cell wall of lysed gram negative bacterial organisms. The substance has a broad spectrum of biological activities affecting humoral and cellular host mediation systems and has been extensively reviewed.[73] Endotoxin may trigger the activation of complement and coagulation pathways, *in vitro* or *in vivo*, and has profound effects on platelets, neutrophils, macrophages/monocytes, endothelial cells, mast cells and basophils.[73] The interrelationships of these humoral and cellular pathways in the pathogenesis of disease are complex.

Pasteurella haemolytica has a major role in the bovine respiratory disease (BRD) complex. This gram negative bacterium produces an endotoxin and cytotoxin during its log phase growth, both of which may be important in the pathogenesis of the pulmonary lesions which are hallmarks of the disease. Neutrophil depletion experiments have affirmed the necessity for neutrophils in pneumonic pasteurellosis.[129] Because endotoxins from gram negative

bacteria are similar in composition and biological effects, the potential role of endotoxin in the pathogenesis of pneumonic pasteurellosis was investigated by comparing the effects of endotoxin (*Escherichia coli* 055:B5) administered intravenously and intratracheally to anesthetized neonatal calves. While intravenous endotoxin was rapidly fatal, airway administration of identical quantities caused no significant physiologic or pathologic changes.[188]

The pathogenesis of neutrophil-mediated injury is thought to be related to the production of oxygen-derived free radicals ($^1O_2$, H_2O_2, O_2^-,OH^-), release of enzymes from stores of intracytoplasmic granules and production of arachidonic acid metabolites (prostaglandins and leukotrienes).[1-3] Results of *in vitro* studies have indicated that exposure of human neutrophils to trace amounts of endotoxin enhanced neutrophil oxidative metabolism, specifically the production of superoxide anion.[76] The question posed in the present study was whether trace amounts of endotoxin had similar effects on bovine neutrophils. The generation of luminol-enhanced chemiluminescence (LECL) and production of superoxide by stimulated bovine neutrophils were used to investigate the effects of endotoxin on neutrophil oxidative metabolism.

MATERIALS AND METHODS: Neonatal Holstein bull calves were purchased from a large local dairy herd. The calves upon arrival at MSU received an oral rotavirus and

coronavirus vaccine[a] and intramuscular vitamin E/selenium[b] and vitamin ADE[c] injections upon arrival. The calves were examined twice daily with body temperature, pulse rate, respiratory rate, appetite, attitude, and appearance of feces recorded. The calves were exercised once daily. Several blood samples were drawn during the initial week of acclimation for total and differential white blood cell (WBC) counts, which were used as an indicator of the health of the calf, and as a guide to the amounts of blood (and neutrophils) needed for completion of the protocols. Several aliquots of serum were harvested for use in the preparation of autologous opsonized zymosan (OZ). The calves were used for a maximum of 3 weeks.

Blood samples were collected in syringes containing acetate-citrate-dextrose (ACD) anticoagulant, and neutrophils were isolated by differential centrifugation and hypotonic lysis. The final isolates of cells were maintained in calcium- and magnesium-free Hank's balanced salt solutions (HBSS) as previously described in chapter 1. Test tubes containing the isolated cells were maintained in crushed ice until used in the experiments.

CHEMILUMINESCENCE: Luminol-enhanced chemiluminescence (LECL) was quantitated [in counts per minute (CPM)] in a Beckman LS 7500 liquid scintillation counter[d] programmed

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[a]Calf Guard, Norden Laboratories, Lincoln, NE.
[b]Bo-Se, Schering Corporation, Kenilworth, NJ.
[c]Vitamin ADE Injection, W.A. Butler Co., Columbus, OH.
[d]Beckman Instrument Company, Fullerton, Ca.

to count at 0.1 minute intervals for 15 minutes in an in-coincidence mode. A 70 uM (end point concentration) of luminol was used to enhance the generation of CL. A series of agonists, which stimulate neutrophil LECL by different mechanisms, were used including latex particles (LP), phorbol ester (PMA), calcium ionophore (CI) and opsonized zymosan (OZ). The methods of agonist preparation and quantities used to stimulate neutrophil LECL and superoxide production were as described previously (Chapter 1). For analysis of data, the five data points (counts) for each minute of elapsed time were averaged and log transformed. Luminol-enhanced CL peak counts, slope responses, times of initial response and times of peak response were calculated as previously described in Chapter 1 and recorded for statistical analyses.

SUPEROXIDE PRODUCTION: The production of superoxide anion by stimulated neutrophils was quantitated by the superoxide dismutase-inhibitable reduction of ferricytochrome C[53,54] as previously described (Chapter 1). The series of agonists used to induce the production of superoxide consisted of PMA, CI, and OZ. Unstimulated control cells were included in each assay. The amounts of superoxide produced by the stimulated cells were calculated in nM of O_2-/2 X 10^6 cells/30 minutes and were recorded for statistical analyses.

PREPARATION OF ENDOTOXIN: Phenol-extracted *Escherichia coli* 055:B5 endotoxin (EC:LPS) was obtained from commercially available sources[e]. Phenol-extracted *Pasteurella haemolytica* endotoxin (PH:LPS) was generously supplied by Dr. Robert Walker, head of the bacteriology/mycology section of the Animal Health Diagnostic Laboratory at Michigan State University. The disposable capped polystyrene test tubes[f] used in the preparation of both endotoxins were those recommended by Cape Cod Associates, Woods Hole, MA. for the preparation and subsequent quantitation of endotoxin solutions, since they contained virtually no contaminating endotoxin. Two milligrams of PH:LPS and EC:LPS were diluted in 5 ml of endotoxin-free water. The test tubes were capped, and the preparations of PH:LPS and EC:LPS mixed by a vortex mixer at room temperature for one hour. To enhance solubility, the capped tubes were refrigerated at 4 C for 24 hours and again mixed at room temperature for 30 minutes. These were designated as stock solutions for both endotoxins.

Dilutions, using endotoxin-free water, of each stock solution were made into polystyrene test tubes to an approximate concentration of 1000 ng per ml. The tubes were capped and 10 ml aliquots of each endotoxin solution were packed in dry ice and shipped to Cape Cod Associates in Woods Hole, MA. for endotoxin quantitation by turbidometric

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[e]Sigma Chemical Company, St. Louis, MO.
[f]Falcon Tube #2095, Falcon, Oxnard, CA.

Limulus amebocyte assay. An aliquot of prepared HBSS with Ca^{2+} and Mg^{2+}, used in LECL and superoxide production protocols, was also submitted for quantitation of contaminating endotoxin. Upon receipt of the quantitative report, final dilutions of each endotoxin stock solution were made to ensure quantitatively equal potency (1000 ng per ml). The sample of HBSS submitted was reported to contain 0.001 ng of endotoxin per ml. These preparations, designated "working dilutions" were stored in capped endotoxin-free borosilicate glass liquid scintillation vials at 4 C. This method of storage of the working dilutions was suggested by Cape Cod Associates as being able to preserve the stability of LPS preparations for as long as two years without significant loss of potency. The scintillation vials containing the working dilutions were mixed by vortex mixer at room temperature just prior to addition of the selected quantities of endotoxin to the neutrophil suspensions.

EXPERIMENTAL DESIGN: It was assumed that the limit of maximal cell viability was 6 hours from the time of blood withdrawal, therefore all of the LECL and superoxide production experiments were completed within that time. The sequence of agonists used to stimulate neutrophils was changed daily to ensure that the time which had elapsed after blood withdrawal did not influence the responses of the neutrophils. In this series of experiments, the responses of unstimulated control cells exposed to the

selected concentrations of each endotoxin and those unexposed to endotoxin were quantitated and included as an analysis variable in each assay. Neutrophils and the selected endotoxin concentrations were incubated for 1 hour at 37 C. The four trace quantities of each endotoxin which were evaluated included 10, 20, 50, and 100 ng/ml. These quantities represented the amounts to which the neutrophils were exposed during incubation. During the hour of incubation, LECL control assays (unexposed cells) were performed using the sequence of agonists selected for that day's experiments. After incubation with endotoxin for 1 hour, the endotoxinexposed neutrophils were used in LECL protocols (using the same agonist sequence) and the superoxide protocols. Only one concentration of endotoxin could be evaluated in each day's experiments. The sequence of endotoxin concentrations investigated each week was randomized. A total of 6 separate experiments were performed for each of the four concentrations of endotoxin.

STATISTICAL DESIGN: For studies of the effects of PH:LPS and EC:LPS on neutrophil responses, superoxide production, LECL (peak and slope), and initial and peak response time data were analyzed by a computer based statistical program using three-way factorial analysis of variance (ANOVAs).[g] Factors evaluated included: 1) the variability in the results of experiments which could be attributed to the

[g]SAS Institute Inc., Cary, NC.

responses (to all agonists) of neutrophils from individual calves (termed calf variability), 2) the differences in neutrophil responses induced by the different agonists, 3) the differences in the responses of stimulated neutrophils induced by exposure to different endotoxin concentrations, and 4) the interactions of these factors. The least significant difference $[LSD\ (T)]^g$ test was used to compare means of the treatment group(s) when there was significance ($P \leq 0.05$) in the individual analysis factors (variables).

To determine the overall effects of endotoxin exposure on neutrophil responses encompassing the responses to all agonists, a modified three-way factorial ANOVA was used in which treatments (control, PH:LPS-exposed, EC:LPS-exposed) and responses to agonists were paralleled. In this design, interaction between treatment and agonist could not be determined. The analyses of PRT and IRT used only a modified three-way ANOVA for determination of overall significance, since it was assumed that endotoxin treatment and/or agonist exposure would induce the principal effects on neutrophil responses. It was also assumed that neither a decreased nor increased response time, alone, would play a significant role in the generation of LECL.

RESULTS: Analyses of the neutrophil peak LECL response data (peak response 1, Table 2.1), indicated that there were no significant effects due to calves or to endotoxin levels, but there were significant differences in the peak

Table 2.1. Comparative Endotoxin Studies: Effects of Pasteurella haemolytica and Escherichia coli Endotoxins on the Oxidative Metabolism of Bovine Neutrophils.

PROTOCOL	A LEVEL	B LEVEL	C LEVEL	INTERACTION	FIGURE
Peak Response 1*	NS	Sign	NS	A*B, B*C	3-1;
Peak Response 2**	Sign	Sign	NS	NS	
Slope Response 1*	NS	Sign	NS	NS	3-2;
Slope Response 2**	NS	Sign	NS	NS	
Initial Response Time**	NS	Sign	NS	NS	3-3;
Peak Response Time**	NS	Sign	NS	NS	
Superoxide 1*	Sign	Sign	NS	A*B, A*C	3-4;
Superoxide 2**	Sign	Sign	NS	NS	

*Three-way factorial ANOVA; Calves=3; A=calf; B=agonist; C=endotoxin concentration; **Modified three-way factorial ANOVA; A=endotoxin treatment; B=agonist; C=endotoxin concentration; Where significant at $P \leq 0.05$, comparison of means was by the least-significant-difference test; If significant interaction, groups with interaction are given; Sign=significant at $P \leq 0.05$; NS=not significant; Figures=graphs of statistical analyses;

responses induced by different agonists, with significant interactions between calves and agonists, and between calves and endotoxin levels. Modified three-way analyses (peak response 2, Table 2.1) in the overall design revealed significant differences in neutrophil responses due to endotoxin exposure with both endotoxins significantly decreasing cell responses. There were also significant effects due to agonist, but no effect of endotoxin level, and no interaction. Both endotoxins significantly reduced the LECL peak responses of neutrophils to PMA and CI (Figure 2.1).

Analyses of the neutrophil slope responses (slope response 1, Table 2.1) showed no calf effect, a significant agonist effect, and no effect of endotoxin level, with no interaction. Modified-three way analysis (slope response 2, Table 2.1) showed no effect of endotoxin exposure, a significant effect due to agonist, and no effect of endotoxin level with no interaction. Both endotoxins significantly decreased the neutrophil slope response to PMA whereas only EC:LPS significantly decreased the response to CI over control cells (Figure 2.2). With the exception of this latter effect, the peak responses and slope responses paralleled each other.

When the initial response times (IRT) of stimulated neutrophils were analyzed, there was no significant effects of endotoxin incubation or endotoxin concentrations, but there

Figure 2.1. Comparison of the LECL Peak Responses of Bovine Neutrophils Exposed to Trace Quantities of *Pasteurella haemolytica* Endotoxin (PH:LPS) and *Escherichia coli* Endotoxin (EC:LPS) upon Stimulation by Soluble and Particulate Agonists.

Analyses by three-way factorial ANOVA with comparison of significant ($P \leq 0.05$) means by the least-significant-difference (LSD) Test. Each bar represents the means of each treatment group with a standard error bar. PMA=phorbol ester; CI=calcium ionophore; OZ=opsonized zymosan; UC=unstimulated cells; All comparisons were made to control cells. Horizontal brackets with a centered asterisk (*) enclose treatment groups that differ significantly from control cells. Horizontal brackets with a subtitle NS enclose groups with no significant differences. N=number of experiments.

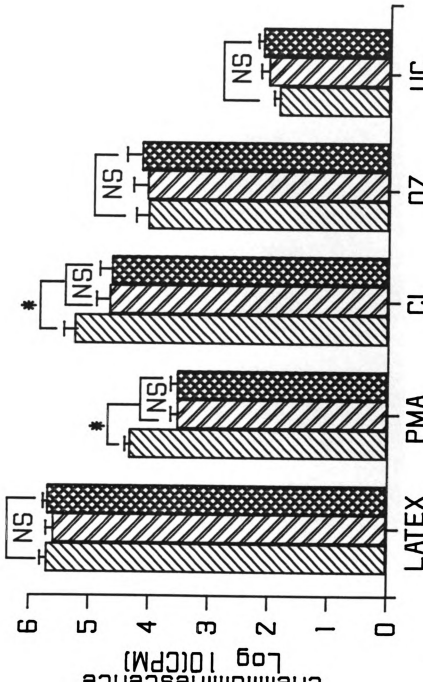
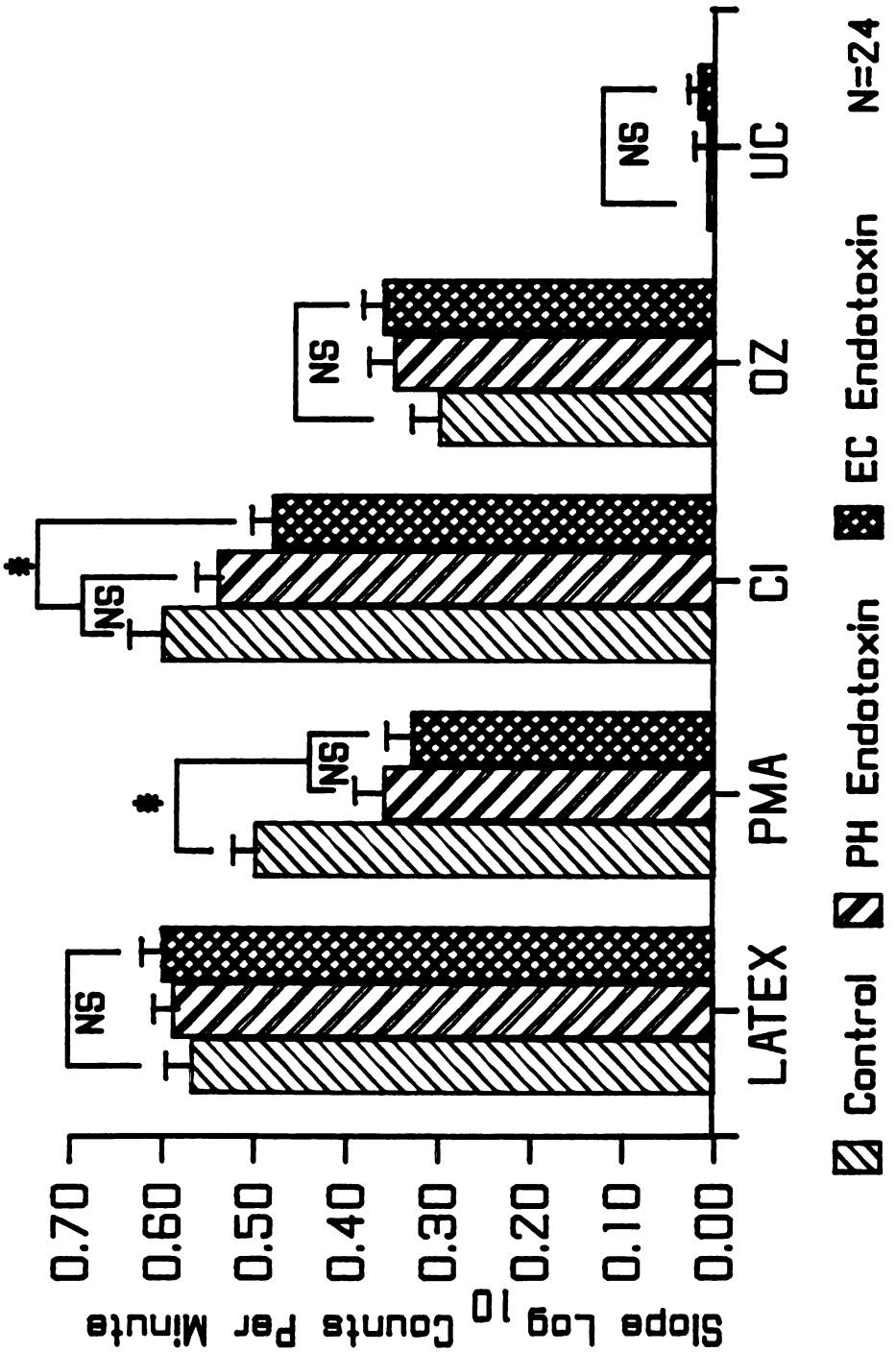

Luminol-enhanced chemiluminescence Log 10(CPM)
0
1
2
3
4
5
6
NS
*
NS
*
NS
NS
LATEX
PMA
CI
OZ
UC
Control
PH Endotoxin
EC Endotoxin N=24

Figure 2.2. Comparison of the LECL Slope Responses of Bovine Neutrophils Exposed to Trace Quantities of PH:LPS and EC:LPS upon Stimulation by Soluble and Particulate Agonists.

Analyses by three-way factorial ANOVA and LSD. Each bar represents the means of each treatment group with a standard error bar. Abbreviations of each treatment group identical to Figure 2.1. All comparisons were made to control cells. Horizontal brackets with a centered asterisk (*) enclose treatment groups that differ significantly. Horizontal brackets with a subtitle NS enclose groups with no significant differences. N=number of experiments.


Slope Log 10 Counts Per Minute
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
NS
NS
NS
NS
*
*
LATEX
PMA
CI
OZ
UC
Control
PH Endotoxin
EC Endotoxin
N=24

were significant differences in the IRT between agonists. There were no interactions. Only the unstimulated cells exposed to PH and EC endotoxins had prolonged IRT, but none of the responses to other agonists differed significantly from control cells (Table 2.1, Figure 2.3).

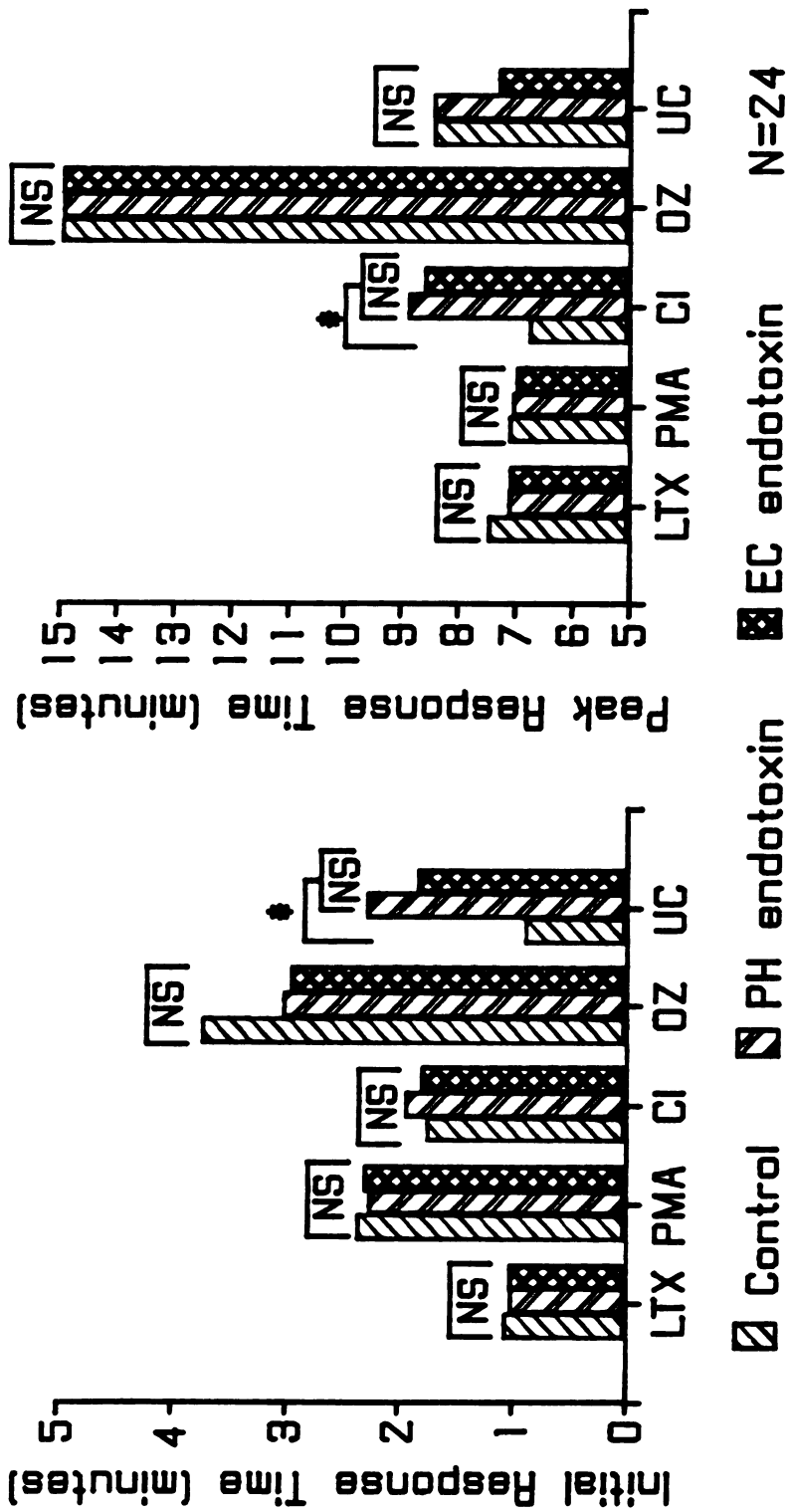
Data analysis for endotoxin effects on neutrophil peak response times (PRT) revealed no significance due to endotoxin incubation or endotoxin concentration and no interaction. Only the agonists induced a significant effect, with CI-stimulated cells showing prolonged PRT after incubation with both endotoxins (Table 2.1, Figure 2.3).

The results of three-way factorial ANOVA analyses of superoxide production data (superoxide 1, Table 2.1) showed significant differences in the responses of neutrophils from individual calves, and significance due to agonists, but none due to endotoxin concentrations. There was, however, a significant interaction between calves and endotoxin level. The neutrophils from calf A2 produced significantly more superoxide than those from calves A1 and A3.

Modified three-way analyses (superoxide 2, Table 2.1) revealed a significant effect of both endotoxins on neutrophil superoxide production, but there was no effect of endotoxin concentration. The different agonists stimulated the production of significantly different quantities of superoxide after endotoxin exposure. The superoxide production of PMA-stimulated cells exposed to both endotoxins increased

Figure 2.3. Comparison of the LECL Initial and Peak Response Time Responses of Bovine Neutrophils Exposed to Trace Quantities of PH:LPS and EC:LPS upon Stimulation by Soluble and Particulate Agonists.

Analyses by three-way factorial ANOVA and LSD. Each bar represents the means of each treatment group with a standard error bar. LTX=latex particles; all other abbreviations for treatment groups identical to Figures 2.1 and 2.2. All comparisons were made to control cells. Horizonal brackets with a centered asterisk (*) enclose treatment groups that differ significantly. Horizontal brackets with a subtitle NS enclose groups with no significant differences. N=number of experiments.

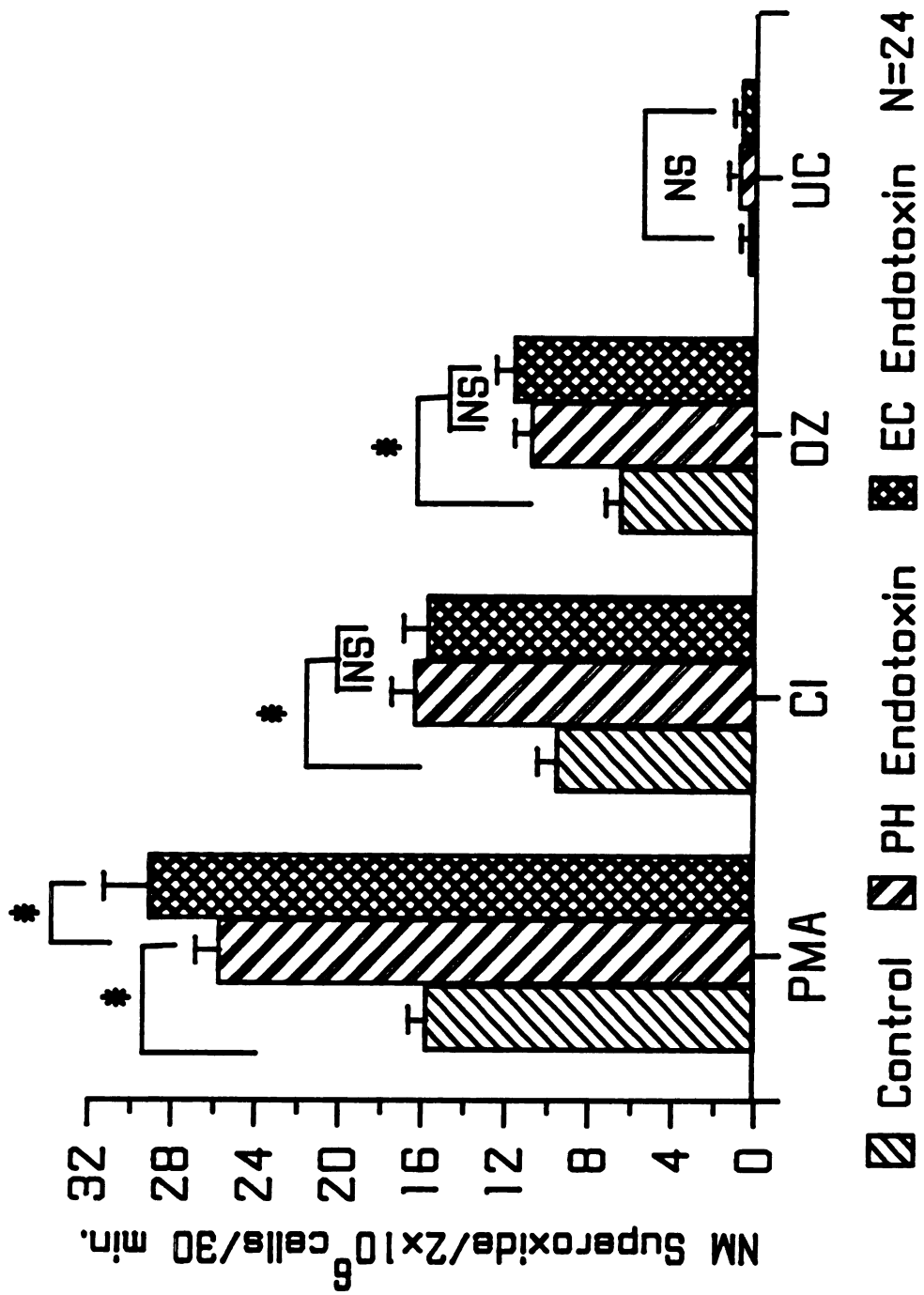

Initial Response Time (minutes)
Peak Response Time (minutes)
LTX PMA CI OZ UC
Control
PH endotoxin
EC endotoxin
N=24
NS
*

significantly, but the responses of EC:LPS-exposed cells were also significantly higher than those exposed to PH:LPS. The CI- and OZ-stimulated responses of cells exposed to both endotoxins also increased significantly, but there were no significant differences between the effects of the endotoxins (Figure 2.4).

DISCUSSION AND CONCLUSIONS: The responses of neutrophils to endotoxin, *in vitro*, have been reviewed[73,85], and the specific responses to trace quantities of endotoxin (< 100 ng) investigated.[76,77,93] These data from my studies represent the first reports of the effects of trace endotoxin concentrations on the oxidative metabolism of bovine neutrophils and the first comparison of the effects of EC:LPS and PH:LPS. Since the responses of neutrophils reported previously[76,93] required the incubation of the cells with endotoxin for a minimum of 30 minutes and gave a maximal effect after incubation for 60 minutes, only the latter incubation time was used in our protocols. I chose to use the series of agonists and agonist concentrations previously defined (Chapter 1) to determine the effects of endotoxin exposure. Previous reports[76,93] had shown that exposure of neutrophils to trace quantities of endotoxin caused significant increases in superoxide production when the neutrophils were stimulated with PMA and OZ. In addition to PMA and OZ, my experiments also used latex and CI as agonists to determine the effects of endotoxin exposure.

Figure 2.4. Comparison of the Superoxide Production of Bovine Neutrophils Exposed to Trace Quantities of PH:LPS and EC:LPS upon Stimulation by Soluble and Particulate Agonists.

Analyses by three-way factorial ANOVA and LSD. Each bar represents the means of each treatment group with a standard error bar. Abbreviations for treatment groups identical to figures 2.1, 2.2 and 2.3. All comparisons were made to control cells. Horizontal brackets with a centered asterisk (*) enclose treatment groups that differ significantly. Horizontal brackets with a subtitle NS enclose groups with no significant differences. N=number of experiments.


NM Superoxide/2x10^6 cells/30 min.
0
4
8
12
16
20
24
28
32
PMA
CI
OZ
UC
NS
NS
*
*
*
Control
PH Endotoxin
EC Endotoxin
N=24

Since these agonists all trigger neutrophil oxidative metabolism by different mechanisms, I hoped to selectively determine which pathway(s) might be enhanced by exposure to endotoxin. Since endotoxin increases superoxide production to selected agonists, I reasoned that the compound might induce similar effects on generation of LECL by stimulated neutrophils. Therefore, my investigation was expanded to evaluate the effects of endotoxin on LECL. Experiments to compare the effects of EC:LPS and PH:LPS on bovine neutrophil oxidative metabolism were designed to: 1) resolve the premise that the biological effects of endotoxins vary little between bacterial species; 2) determine whether the effects of PH:LPS were specific for bovine neutrophils; 3) explore the hypothesis that exposure of bovine neutrophils to trace quantities of LPS might "prime" the cells to produce increased quantities of luminol-reactive entities (reactive oxygen species) including superoxide anion, after exposure to agonists; 4) determine whether these endotoxin effects might be localized to a specific pathway or mechanism of neutrophil activation based on agonist responses; and 5) determine whether these effects might have a role in the pathogenesis of pneumonic pasteurellosis.

To answer these questions I used techniques and agonists defined in Chapter 1 to investigate bovine neutrophil oxidative metabolism. These agonists were selected because they activate neutrophils by different mechanisms, as

previously discussed. Latex particles activate neutrophils as a function of the size and ionic properties of the particles[187], with membrane perturbation of surface sialic acid residues leading to ionic transport as the initial basis of response[6], followed sequentially by responses activated by particle phagocytosis.[186] Phorbol ester (PMA) activates neutrophils by direct stimulation of protein kinase C, an enzyme intimately involved in the activation of the "respiratory burst" enzyme, NADPH-oxidase, as well as in other sequential cellular functions.[19-21] Calcium ionophore (CI) induces neutrophil activation by the transport of extracellular Ca^{2+} into the cytosol where it has a major role as a second messenger.[4,6] The increase in intracellular Ca^{2+} may trigger the activation of several cytosolic and membrane-bound Ca^{2+}-dependent enzymes known to be important in cellular transduction. Opsonized zymosan activates neutrophils by mechanisms dependent on the activation of phospholipase-A_2 and the presence of cytosolic calcium.[50] While the use of OZ has been suggested to initiate the polyphosphoinositide cascade, the activation of cells by OZ is independent of protein kinase C.[50]

Experiments described in chapter 1, in which the oxidative responses of bovine neutrophils were studied, revealed marked day-to-day variability in the neutrophil responses of individual calves as well as differences between individual calves. To assess the possible effects of variability

due to calf factors, three-way factorial ANOVAs were used to examine the responses of neutrophils. In these analyses, the interaction between calf factors and other variables could be determined. In the protocols comparing PH:LPS and EC:LPS, only the analyses of superoxide production data (table 2.1) had significant differences in neutrophil responses due to the calf from which the neutrophils originated. Only the analyses of data for LECL peak response and superoxide production revealed significant interactions of calf factors with other factors.

The "priming" phenomenon has been described as the enhanced production of superoxide anion after incubation with trace levels of endotoxin (< 100 ng).[76] In my studies I examined the effects of similar trace levels of endotoxin on LECL generation and superoxide production of bovine neutrophils. The studies of the effects of endotoxin on LECL examined several neutrophil function parameters including peak response, slope response, times of initial response, and times of peak response. Examples of "priming" which might be anticipated using these parameters of neutrophil responses should hypothetically include: 1) an increase in the neutrophil peak response; 2) an increased slope response; 3) a decreased or faster time of initial response; and 4) an earlier time of peak response. For superoxide, production "priming" should enhance the production of superoxide anion. If the hypotheses that were supported by the

increased production of superoxide by human neutrophils after stimulation by PMA and OZ are also true for bovine neutrophils, then similar changes (as above) should occur in the LECL parameters and superoxide production after stimulation by these agonists.

The molecular basis of the "priming" effect was suggested to be: 1) alterations of the plasma membrane so that contact or binding of stimuli might be greater; 2) that events coupling stimulus-receptor interaction leading to activation of the NADPH-oxidase might activate more enzyme; or 3) the conformation of or placement of the enzyme in the membrane might be changed so that it expresses higher activity.[76] Since it has been shown that neutrophils have the capability to metabolize endotoxin, it was postulated that the lipid A fractions might be metabolized to form fatty acids, which can induce superoxide release, and directly activate protein kinase C (PKC).[76] It was suggested that the time and temperature requirements were consistent with the formation of a metabolite which might directly activate PKC or stimulate increased polyphosphoinositide metabolism and turnover indirectly activating PKC.[20]

There was a significant effect of endotoxin-exposure, on LECL peak responses, seen with both endotoxins but no effect of endotoxin concentration. Both endotoxins significantly reduced the peak responses to PMA and CI but had no

significant effect on the neutrophil responses to latex and to OZ. The slope responses paralleled the peak responses in that the responses to PMA and CI were also significantly reduced, but for the latter only the response to EC:LPS was significantly reduced from control cells. In support of the decreased peak and slope responses to CI, the time of peak response was significantly prolonged. In sharp contrast to the LECL response data, exposure of neutrophils to both endotoxins induced significant enhancement of superoxide production after stimulation by PMA, CI, and OZ. The interpretation of these diametrical differences in neutrophil responses to the same agonists is difficult. Under the strict definition of "priming", only the superoxide production responses would fulfill the criteria discussed previously.

The inability to correlate the results of the LECL generation and superoxide production assays has been addressed in Chapter 1. This is in part due to variable quantities of superoxide anion and other reactive oxygen species produced by each agonist, and the relative contributions of each entity to the LECL response. The superoxide anion was shown to be responsible for a larger part of the LECL peak responses when stimulated by soluble agonists (PMA, CI) than those when stimulated by particulate agonists (latex, OZ). Since the production of superoxide in response to soluble agonists is enhanced by endotoxin exposure, the

production of other luminol-reactive entities such as $\cdot OH$, $^1O_2$, H_2O_2, and $HOCL^-$ must be markedly inhibited for peak LECL to drop so significantly. Since the catabolism or disposal of superoxide anion is mediated principally through the activity of cytosolic superoxide dismutase, decreased activity would decrease the amounts of H_2O_2 formed for disposal by other enzyme systems, such as catalase, the myeloperoxidase-halide system, and the glutathione peroxidase system. If there are increased quantities of hydrogen peroxide produced, an inhibition of the myeloperoxidase enzyme might diminish the production of hypohalous radicals and singlet oxygen, thereby decreasing LECL. Another possible explanation is that the glutathione system might catabolize the formed hydrogen peroxide at an increased rate. Since it has been shown that bovine neutrophils, when stimulated, have a marked activation of the hexose monophosphate shunt, and since the production of reducing equivalents necessary for activation of the respiratory burst enzyme NADPH-oxidase are intimately tied to the glutathione system and the HMP shunt, it is possible that this might explain not only the increased superoxide production but also the decreased availability of hydrogen peroxide for use by other enzymes.

Since the mechanisms of induction of neutrophil oxidative metabolism by particulates follow different intracellular pathways than for soluble agonists[21,50], it is

possible that there is also a selective enhancement of the pathways for superoxide production and selective inhibition of others involved in the generation of LECL by particulates. Since there was enhancement of superoxide production after exposure to endotoxin and stimulation by OZ, yet no significant increase in LECL, this suggests that the lack of correlation between the two assays might be explained by: 1) the relative contribution of superoxide anion to the LECL response to particulates; and/or 2) a less significant inhibition of the production of other luminol-reactive entities, perhaps related to the different pathways of neutrophil transduction used by particulate agonists.

The decrease in LECL responses and enhancement of superoxide production, after endotoxin exposure, to stimulation by PMA suggests three hypotheses: 1) that either PKC itself and/or its substrates are altered by endotoxin; 2) that changes in membrane permeability allow less penetration of PMA into the cytosol; or 3) that endotoxin alters enzymes distal to PKC which are necessary for the selective induction of oxidative metabolism.

Hypotheses for the decreased LECL responses and enhancement of superoxide production, after endotoxin exposure, to CI must center upon: 1) altered Ca^{2+} transport, either reduced or enhanced, perhaps due to altered plasma membrane permeability; or 2) alteration of intracytoplasmic Ca^{2+}

dependent enzymes including PKC, calpain, phospholipase A_2, phospholipase C, and calmodulin, perhaps selectively affecting production of other second messengers.

Hypotheses for the the increase in superoxide production, after endotoxin exposure, to OZ include: 1) alteration of surface enzymes facilitating particle/surface interaction; 2) up-regulation of surface complement or immunoglobulin receptors; or 3) selective enhancement of enzymes (phospholipase-A_2) involved in the cell response to particulates. These postulates detail not only the complexity of neutrophil transduction mechanisms but also the difficulties in correlating the neutrophil responses between assays, and between species.

In conclusion, these studies have determined: 1) that the exposure of neutrophils to endotoxin has profound effects on neutrophil oxidative metabolism but results are dependent upon the assay procedure, the agonist(s) used, and the species from which the neutrophils were obtained; 2) that there were no significant differences in the responses of bovine neutrophils after incubation with PH:LPS or EC:LPS, therefore ruling out a species-specific effect; 3) that there were no significant differences in the effects of four different concentrations of endotoxin on the responses of exposed neutrophils; and 4) that these data support the contention that endotoxins vary little in their biological effects, differing only in their potency. In

the latter conclusion, potential variability between endotoxins was eliminated by accurate quantitation of endotoxin concentrations. These data also support the principal hypothesis that exposure of (bovine) neutrophils to trace concentrations (10-100 ng) of endotoxin may "prime" them to produce increased quantities of superoxide anion after stimulation by PMA, OZ, and additionally by CI and that this response was irrespective of the species (PH or EC) from which the endotoxin originated.

Therefore, the presence of small quantities of endotoxin released by dying gram negative bacteria, such as *Pasteurella haemolytica*, may facilitate pulmonary injury via release of superoxide anion from "primed" bovine neutrophils. Since complement activation did not play a role in these *in vitro* studies (serum-free media), this "priming" effect was not due to the indirect action of complement factors.

It is difficult to equate the results of my studies with those of Confer and Simons[159] who used vastly different concentrations of *Pasteurella haemolytica* LPS to determine the *in vitro* effects on bovine neutrophils. They concluded: 1) that PH:LPS was not directly toxic to neutrophils; 2) that PH:LPS had little effect on the random migration of bovine neutrophils; 3) that exposure to PH:LPS enhanced the NBT reduction of exposed cells; 4) that low

(2.5 ug/10^6 cells) and high (65 ug/10^6 cells) concentrations decreased the phagocytosis of labeled *Staphylococcus aureus*; and 5) that moderate quantities (5-25 ug/10^6 cells) increased the phagocytosis of labeled *Staphylococcus aureus*. While trypan blue due exclusion tests were not performed in our experiments after endotoxin incubation, the enhancement of superoxide production suggests that the neutrophil possessed fully functional metabolic pathways. Since nitroblue tetrazolium reduction is a quantitative measure of superoxide production, the enhanced NBT reduction reported would correlate well with our results of enhanced production of superoxide anion. Since we did not perform random migration studies nor radiolabeled staphylococcal ingestion studies, no correlations are possible between the results of my and their experiments. While their findings that staphylococcal ingestion was enhanced by "moderate" quantities of PH:LPS again provide no correlates to my studies, their data suggest that specific levels of endotoxin facilitate the phagocytosis of bacteria. Whether this response is specific to staphylococci or whether similar responses to *Pasteurella* would also be seen remains to be investigated. There have been no other reports in the veterinary literature which have reported the *in vitro* effects of PH:LPS on the oxidative metabolism of bovine neutrophils.

Future studies should involve the exploration of the effect of endotoxins on the cell responses to the *Pasteurella* organism *in vitro* and *in vivo* with the former encompassing studies of oxidative metabolism and enzyme release and the latter the airway administration of endotoxin followed by variable numbers of *Pasteurella* organisms.

CHAPTER 3: ENZYME RELEASE BY BOVINE NEUTROPHILS

INTRODUCTION: The pathogenesis of neutrophil-mediated injury involves three major mechanisms: the release of reactive oxygen species, the secretion or release of enzymes from cytoplasmic granules, and the production of arachidonic acid metabolites.[1-3] The importance of neutrophils in the pathogenesis of pneumonic pasteurellosis of cattle has been identified by neutrophil depletion of neonatal calves[129], which prevented lung injury after intratracheal inoculation of virulent *Pasteurella* organisms. Although many of the mechanisms of neutrophil activation leading to tissue injury are understood, there is no unifying theory as to which neutrophil product(s) is/are responsible. Cyclooxygenase inhibitors and polyethylene glycol (PEG) bound catalase have not lessened the severity of experimentally-induced *Pasteurella* pneumonia in calves. (unreported data)

Ruminant (bovine) neutrophils are unique, and possess three distinct cytoplasmic granule subtypes which may be differentiated ultrastructurally during myelopoesis by means of peroxidase reactivity.[100-101] The biochemical properties of bovine neutrophils have been delineated[99]

and phylogenetic comparisons have been made to other species.[98] When compared to human neutrophils, bovine neutrophils lack lysozyme, and are relatively deficient in myeloperoxidase and in primary (azurophil) granule component enzymes. They have a large component of secondary (specific) granules and associated enzymes. Since bovine neutrophils are also relatively deficient in catalase, the pathways of hydrogen peroxide (H_2O_2) catabolism are considered to be unique in comparison to neutrophils of other species.[99]

To my knowledge there have been no previous studies which have explored the selective release of granule-associated or cytoplasmic enzymes by bovine neutrophils, either *in vivo*, or *in vitro*. The relationships of these potentially damaging neutrophil products to fulminant pneumonic pasteurellosis are presently undefined. The purpose of this research was to characterize the magnitude of enzyme release by activated bovine neutrophils: first, to a series of defined agonists and subsequently to the *Pasteurella* organism itself.

Marker enzymes for neutrophil cytoplasmic granules have been previously defined and vary among species. For bovine neutrophils, beta-glucuronidase (BG) is the marker for azurophil granules, and vitamin B-12-binding protein (B12BP) is the marker for specific granules.[99] Lactate dehydrogenase (LDH), a cytosolic enzyme, is frequently used as an

indicator of cell death or lysis associated with increased membrane permeability.[53,54]

MATERIALS AND METHODS: Experimental calves were acquired, acclimated, and maintained as previously described. Blood samples were obtained, and neutrophils were isolated by previously described methods. All reagents, analytical grade, were obtained from Sigma[a], and were prepared as previously described. To provide a basis of comparison for future studies, a series of experimental protocols were designed to explore bovine neutrophil enzyme release in response to the soluble agonists phorbol ester (PMA) and calcium ionophore (CI), and to the particulate agonist opsonized zymosan (OZ). Agonist concentrations used in these protocols were determined from those suggested for use in similar experiments with human neutrophils[53,54] and which were defined further in experiments exploring the oxidative metabolism of bovine neutrophils (Chapter 1). The concentrations used in these enzyme release protocols were: PMA, 200 ng/ml, CI 10^{-5} M, and 50 microliters of autologous opsonized zymosan per 2 X 10^{6} neutrophils. For comparative studies, suspensions of *Pasteurella haemolytica* organisms (PHL, PHLO, PHD, PHDO) were prepared as previously described (Chapter 1) after being adjusted to an optical density of 1.60 at 541 nm in a double beam spectrophotometer[b]. Preparations of

[a]Sigma Chemical Company, St. Louis, MO.
[b]Shimadzu UV-260, Shimadzu Instruments Ltd., Tokyo, Japan

organisms were maintained at room temperature. For stimulation 50 ul of each preparation of bacteria was used per each 2 X 10^6 cells, to give an approximate ratio of 25 bacteria per neutrophil.[133] This particle/cell ratio was nearly identical to that of OZ.[54]

Because of the low levels of BG and LDH in bovine neutrophils, 10 X 10^6 PMNs were used to ensure detectable and reproducible quantities of enzymes (data not shown). For quantitation of B12BP, it was only necessary to use 2 X 10^6 cells. All experiments included positive controls, consisting of equal numbers of cells lysed with 1% triton-X[a], and negative controls. Test tubes (10 X 75 mm) containing PMNs (2 x 10^6 for B12BP, and 10 X 10^6 for LDH and BG), Hanks balanced salt solution with Ca^{2+} and Mg^{2+} (HBSS), and the selected levels of agonists, including positive and negative controls, were placed in a shaking water bath at 37 C for 30 minutes. After agitation, the tubes were centrifuged at 1000 X g for 20 minutes at 4 C. Tubes containing the supernatants were placed in crushed ice until the individual assays were performed.

Beta-glucuronidase release was determined by the hydrolysis of the chromogenic substrate phenolphthalein glucuronide as previously described[53,54], by using an assay kit obtained from Sigma.[a] Aliquots of supernatants from the degranulation assay were incubated in acetate buffer and phenolphthalein glucuronic acid for 18 hours at 37 C.

Linear regression was performed on absorbance data derived from a series of standard samples, was used to obtain a slope and intercept for quantitation of unknown samples. Post-incubation, glycine buffer was added for color development. A double beam spectrophotometer, zeroed at 540 nm with acetate buffer, was used to sequentially read all samples. Values for glucuronic acid controls were subtracted from all other readings. The amounts of BG released in response to the agonists were quantitated as micrograms of phenolphthalein released per 10 X 10^6 cells/ 30 minutes.

For determination of LDH activity, supernatants were added to a prewarmed (37 C) substrate kit[a] in microcuvettes[c] and mixed, and after a 30 second delay the change in absorbance was followed kinetically at 37 C and 340 nm in a double beam spectrophotometer.[b] The changes in absorbance which occurred at five consecutive 1 minute intervals were recorded. The data were averaged and used to calculate the enzyme release in Wacker units. Samples of abnormal serum, generously supplied by Sigma[a], were used to ensure the accuracy of the assay. It was determined that the values (in Wacker units) of the positive controls and the abnormal controls were nearly identical and had excellent reproducibility. Subsequently, only positive controls were performed in each assay.

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[c]Cole Parmer Instrument Co., Chicago, IL.

For determination of B12BP activity[54], cyanocobalamin ^{57}Co-vitamin B_{12} (12 uCi/microgram) was obtained from Amersham[d], diluted in normal saline to a concentration of 5 ng/ml, divided into aliquots, and stored at 4 C in 0.6 ml Eppendorf microcentrifuge tubes[c]. For calculation of specific activity, the radioimmunoassay included total activity and charcoal control (unbound) samples in all assays. Duplicate samples were prepared for all agonists. Sequentially, saline, supernatant, and 1000 pg of ^{57}Co-vitamin B_{12} were added to Eppendorf microcentrifuge tubes (1.6 ml[c]) and mixed using a vortex mixer for 10 seconds. After mixing, 0.4 ml of an equal volume of 5% Norit charcoal and 1% bovine serum albumin (fraction V[a]) was added, and the tubes were again mixed with a vortex mixer. After mixing, the tubes were centifuged for 2 minutes at 12,000 RPM in a centrifuge with a microcentrifuge tube rotor. Equal amounts of supernatant (0.8 ml) were removed from each tube, taking care not to disturb the sedimented charcoal, placed into 10 X 75 mm polypropylene test tubes, and capped. Sample activity (CPM) was read in a Searle Analytic 1185-R gamma counter[x]. Counts derived from exposure of neutrophils to each agonist were recorded in three channels. The readings from the three channels for each of the duplicate samples were averaged to calculate B12BP release.

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[d]Amersham Corp., Arlington Heights, IL.
[e]Searle Analytic, Inc., Des Plaines, IL.

Values were recorded as pg vitamin B_{12} bound/2 X 10^6 neutrophils.

The values for positive controls in each assay were considered to be maximal possible release of enzymes. The maximal values recorded for each agonist were used to determine the percentage (%) release induced by exposure to each agonist.

EXPERIMENTAL DESIGN: For determination of the amounts of BG and LDH released by neutrophils in response to the series of agonists, a total of 30 paired experiments were performed. Twenty experiments were performed to determine the neutrophil BG and LDH responses to the four different treatments of the *Pasteurella* organisms, and results were compared to the responses of unstimulated control cells. Twelve experiments which compared the "same day" BG and LDH release responses to agonists and to the 4 treatments of *Pasteurella* organisms, completed the initial studies. In these studies, the levels of agonists that had been defined in studies of oxidative metabolism were used, with the assumption that these levels would also induce significant enzyme release. For B12BP an initial series of 5 experiments was performed in which the cell requirements and effect of agonist levels were studied (data not shown). These studies confirmed the efficacy of the previously established agonist levels in stimulating specific granule release and the necessity for the use of 2 X 10^6

cells. Subsequently, a series of 10 experiments was conducted which compared the "same day" BG, LDH, and B12BP release responses of neutrophils stimulated by the defined series of agonists and by the four treatments of the *Pasteurella* organisms. In these latter studies, extreme care was taken to assure that each 2 X 10^6 cells received equal numbers of OZ and *Pasteurella* particles per cell. The concentration of soluble agonists, PMA and CI, were the same for all studies.

STATISTICAL ANALYSES: Arcsin square root transformation of the percentage release data was performed prior to statistical analyses. Data were analyzed with a computer based statistical program for one-way analysis of variance (ANOVA, OWA)[f]. Where there were significant effects of treatment in the ANOVA designs at $P \leq 0.05$, least-significant-difference (LSD)[f] tests were used for comparison of means to determine significance between treatment groups.

RESULTS: In the initial comparisons of the responses elicited from neutrophils in response to the agonists, calcium ionophore stimulated the highest secretion and/or release of BG and LDH, with these responses differing significantly from those to PMA, OZ, and unstimulated controls (UC) which were not significantly different (N=30). The responses in these assays are shown graphically in Figure 3.1.

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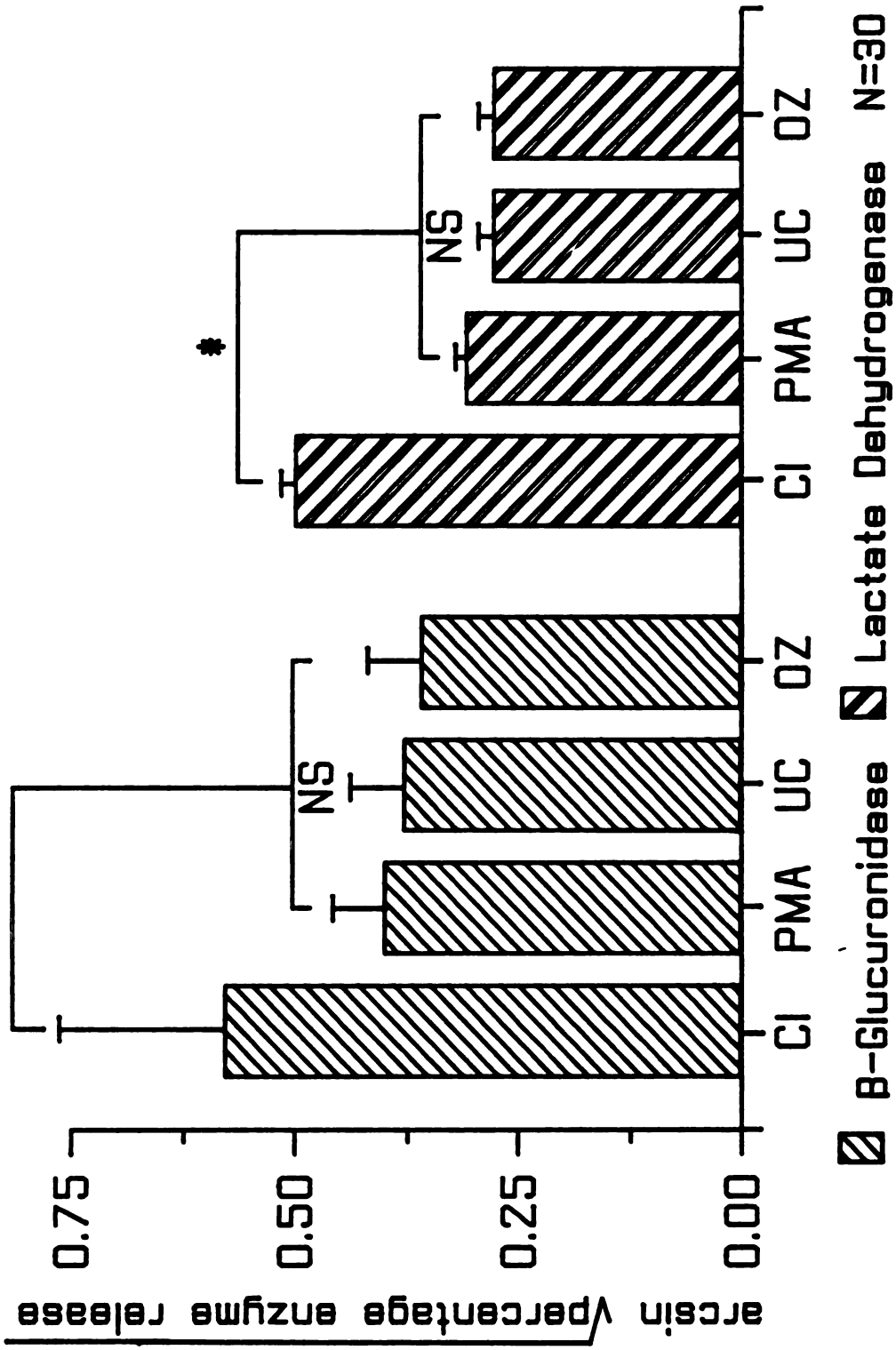
[f]SAS Institute Inc, Cary, NC.

Figure 3.1. Enzyme Release 1: Comparison of the Beta Glucuronidase (BG) and Lactate Dehydrogenase (LDH) Release of Bovine Neutrophils upon Stimulation by Soluble and Particulate Agonists.

Analyses by one-way ANOVA (OWA) with comparison of significant means ($P \leq 0.05$) by the least-significant-difference (LSD) test. Each bar represents the means of each enzyme released in response to each agonist with a standard error bar. PMA=phorbol ester; CI=calcium ionophore; OZ=opsonized zymosan; UC=unstimulated control cells. All percentage release data was transformed by arcsin square root prior to analyses. Horizontal brackets with a centered asterisk (*) enclose treatment groups that differ significantly. Horizontal brackets with a subtitle NS enclose groups with no significant differences. N=number of experiments.

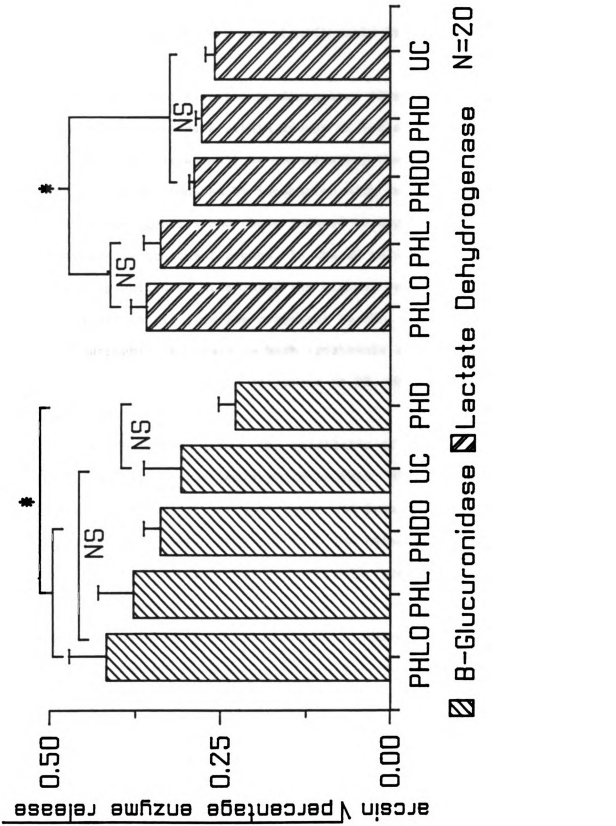

arcsin √percentage enzyme release
0.00
0.25
0.50
0.75
CI
PMA
UC
OZ
NS
*
CI
PMA
UC
OZ
NS
*
B-Glucuronidase
Lactate Dehydrogenase
N=30

The series of experiments designated "Enzyme Release 2" compared the release and/or secretion of BG and LDH induced by the four treatments of the *Pasteurella* organisms and unstimulated control (UC) cells (N=20). These responses showed that there were no significant differences in the quantities of BG released by exposure to live opsonized (PHLO), live (PHL), or dead opsonized (PHDO) when compared to unstimulated control (UC) cells. However, the responses to PHLO, PHL, and PHDO were significantly greater than those to PHD which induced the least BG secretion. PHLO and PHL stimulated the highest release of LDH from neutrophils and amounts were significantly different from the amounts released in response to PHDO and PHD and by UC (Figure 3.2).

Comparisons of the BG and LDH enzyme release responses within the same groups of isolated neutrophils ("same day responses") stimulated by defined agonists and the *Pasteurella* groups (Enzyme Release 3) showed that CI induced the highest (significant) secretion of BG, while the responses to PHLO, PHL, PMA, OZ, PHDO, and of UC were not significantly different. The BG release in response to PHLO, PHL, and PMA was significantly greater than the release in response to PHD. For LDH, CI stimulated a release that was significantly higher than to PHLO, PHL, and PMA. Only the amounts of enzyme released by exposure to PHLO and PHL differed significantly from the PHDO, UC, PHD, and OZ

Figure 3.2. Enzyme Release 2: Comparison of the BG and LDH Release of Bovine Neutrophils upon Stimulation by *Pasteurella haemolytica*.

Analyses by OWA and LSD. Each bar represents the means of each enzyme released in response to each bacterial preparation with a standard error bar. PHLO-live opsonized, PHL-live, PHDO-dead opsonized, PHD-dead *Pasteurella haemolytica* organisms; UC-un-stimulated control cells. Abbreviations for agonist treatment groups identical to figure 3.1. All data was arcsin square root transformed prior to analyses. Horizontal brackets with a centered asterisk (*) enclose treatment groups that differ significantly. Horizontal brackets with a subtitle NS enclose groups with no significant differences. N-number of experiments.


arcsin √percentage enzyme release
0.50
0.25
0.00
*
NS
SN
*
NS
SN
PHLO PHL PHDO UC PHD PHLO PHL PHDO PHD UC
B-Glucuronidase
Lactate Dehydrogenase
N=20

treatment groups which were not significantly different. (Figure 3.3)

The series of 10 experiments designated "Enzyme Release 4" compared the responses within groups of isolated neutrophils ("same day responses") by quantitating the release of BG, LDH, and B12BP induced by incubation with defined concentrations of agonists and those induced by exposure to the *Pasteurella* treatment groups. In the BG release assay, live-opsonized PH stimulated the highest release, differing significantly from the responses to PHL, CI, and PHDO. Neutrophil responses to both treatments also differed significantly from the responses to OZ, PMA, and PHD (Figure 3.4).

In the LDH release assay, the quantities of enzyme released after exposure of neutrophils to PHLO, PHL, and CI differed significantly from each other and from the release stimulated by PMA, PHDO, PHD, and OZ. The release induced by PMA, PHDO, and PHD was also significantly greater than that of UC (Figure 3.5).

In the quantitation of B12BP secretion and/or release, CI and PHLO induced the highest release with the quantities induced by CI significantly different from that induced by PMA. The amounts of B12BP released in response to PHLO, while not significantly different from PMA, differed significantly from those induced by OZ and PHL. The responses to PMA and OZ were statistically similar, with only the

Figure 3.3. Enzyme Release 3: Comparison of the BG and LDH Release by Bovine Neutrophils in Response to Stimulation by Soluble and Particulate Agonists and by *Pasteurella haemolytica*.

Analyses by OWA and LSD. Each bar represents the means of BG and LDH released in response to agonists and bacterial preparations with a standard error bar. Abbreviations for treatment groups identical to Figures 3.1 and 3.2. All data was arcsin square root transformed prior to analyses. Horizontal brackets with a centered asterisk (*) enclose treatment groups that differ significantly. Horizontal brackets with a subtitle NS enclose groups with no significant differences. N=number of experiments.

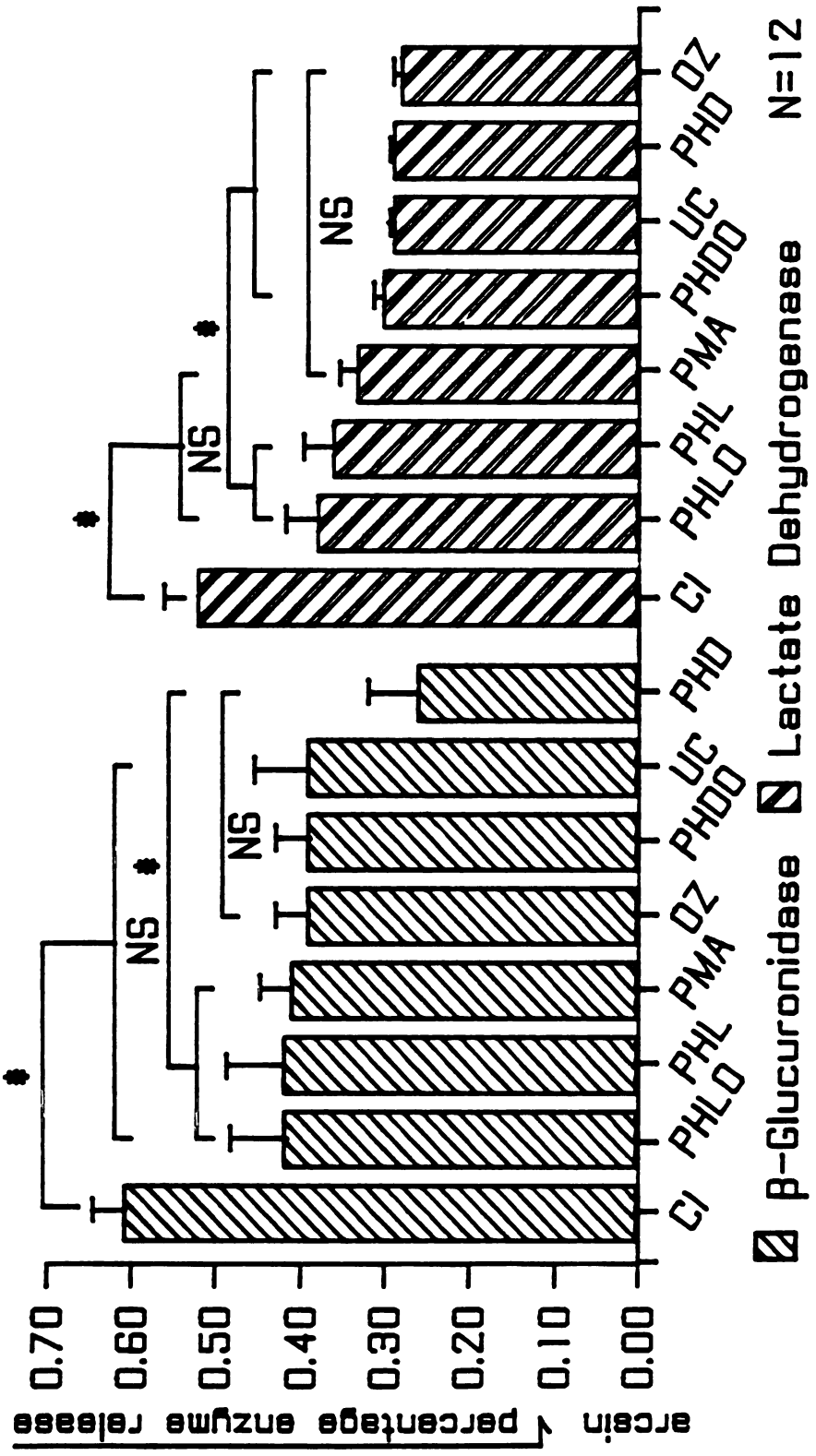

arcsin √ percentage enzyme release
0.00
0.10
0.20
0.30
0.40
0.50
0.60
0.70
β-Glucuronidase
Lactate Dehydrogenase
N=12
CI
PHLO
PHL
PMA
OZ
PHOO
UC
PHO
NS
*

Figure 3.4. Enzyme Release 4: Comparison of the BG, LDH, and Vitamin B_{12}-Binding Protein (B12BP) Release by Bovine Neutrophils in Response to Stimulation by Soluble and Particulate Agonists and by *Pasteurella haemolytica*. Part I: BG Release.

Analyses by OWA and LSD. Each bar represents the means of BG released with a standard error bar. Abbreviations for treatment groups identical to previous figures. All data was arcsin square root transformed prior to analyses. Horizontal brackets with a centered asterisk (*) enclose treatment groups that differ significantly. Horizontal brackets with a subtitle NS enclose groups with no significant differences. N=number of experiments.

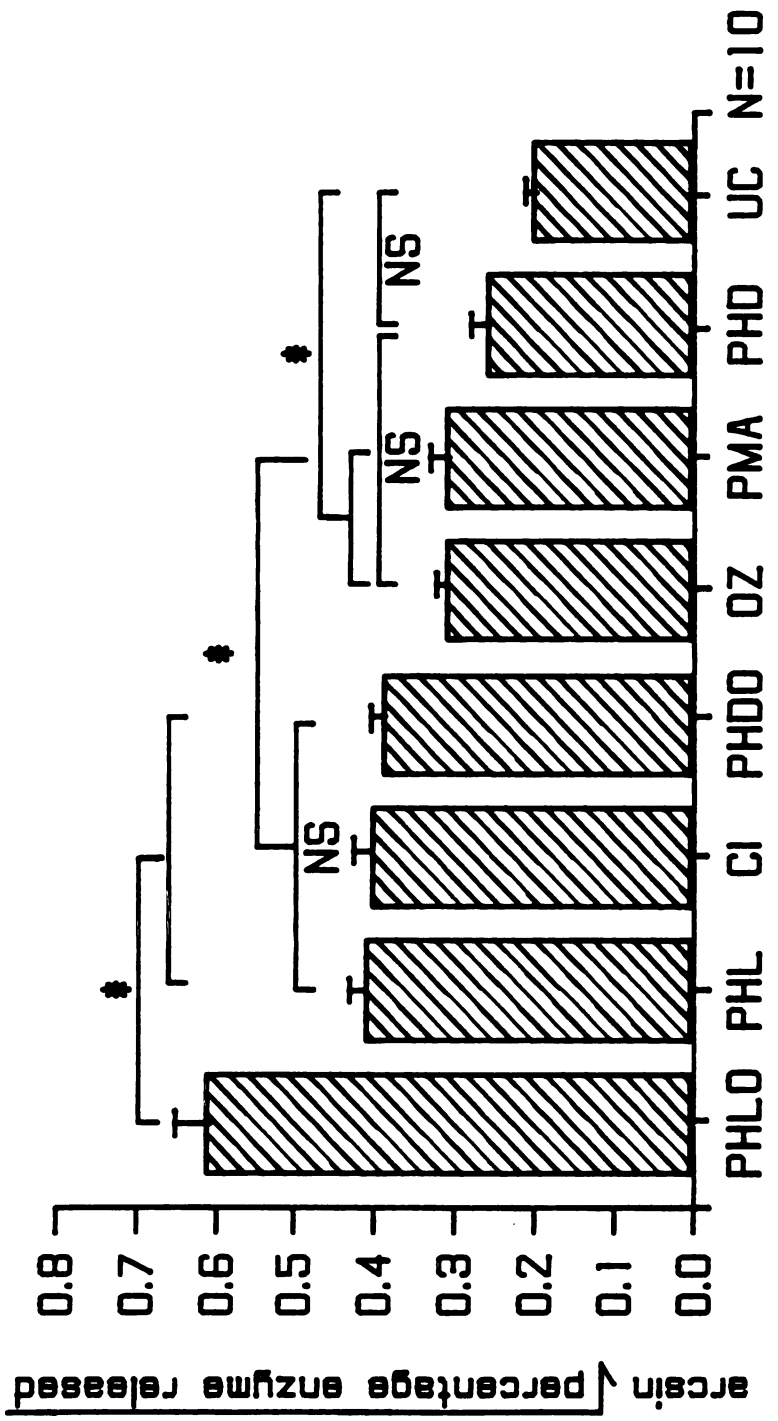
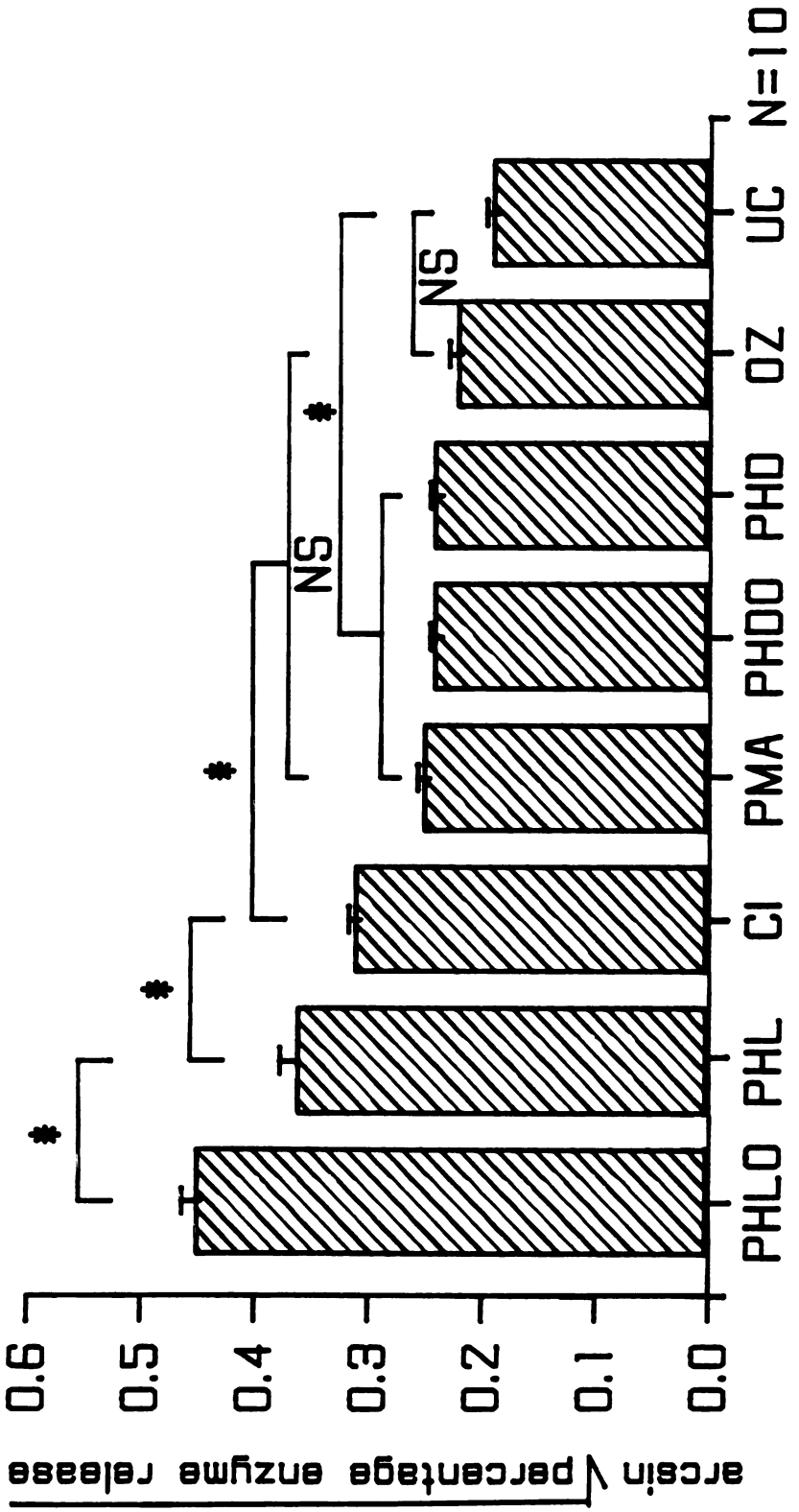

arcsin √ percentage enzyme released
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
PHLO
PHL
CI
PHDO
OZ
PMA
PHD
UC
N=10
*
*
*
NS
NS
NS

Figure 3.5. Enzyme Release 4: Comparison of the BG, LDH, and B12BP Release by Bovine Neutrophils in Response to Stimulation by Soluble and Particulate Agonists and by *Pasteurella haemolytica*. Part II: LDH Release.

Analyses by OWA and LSD. Each bar represents the means of LDH released with a standard error bar. Abbreviations for treatment groups identical to previous figures. All data was arcsin square root transformed prior to analyses. Horizontal brackets with a centered asterisk (*) enclose treatment groups that differ significantly. Horizontal brackets with a subtitle NS enclose groups with no significant differences. N-number of experiments.

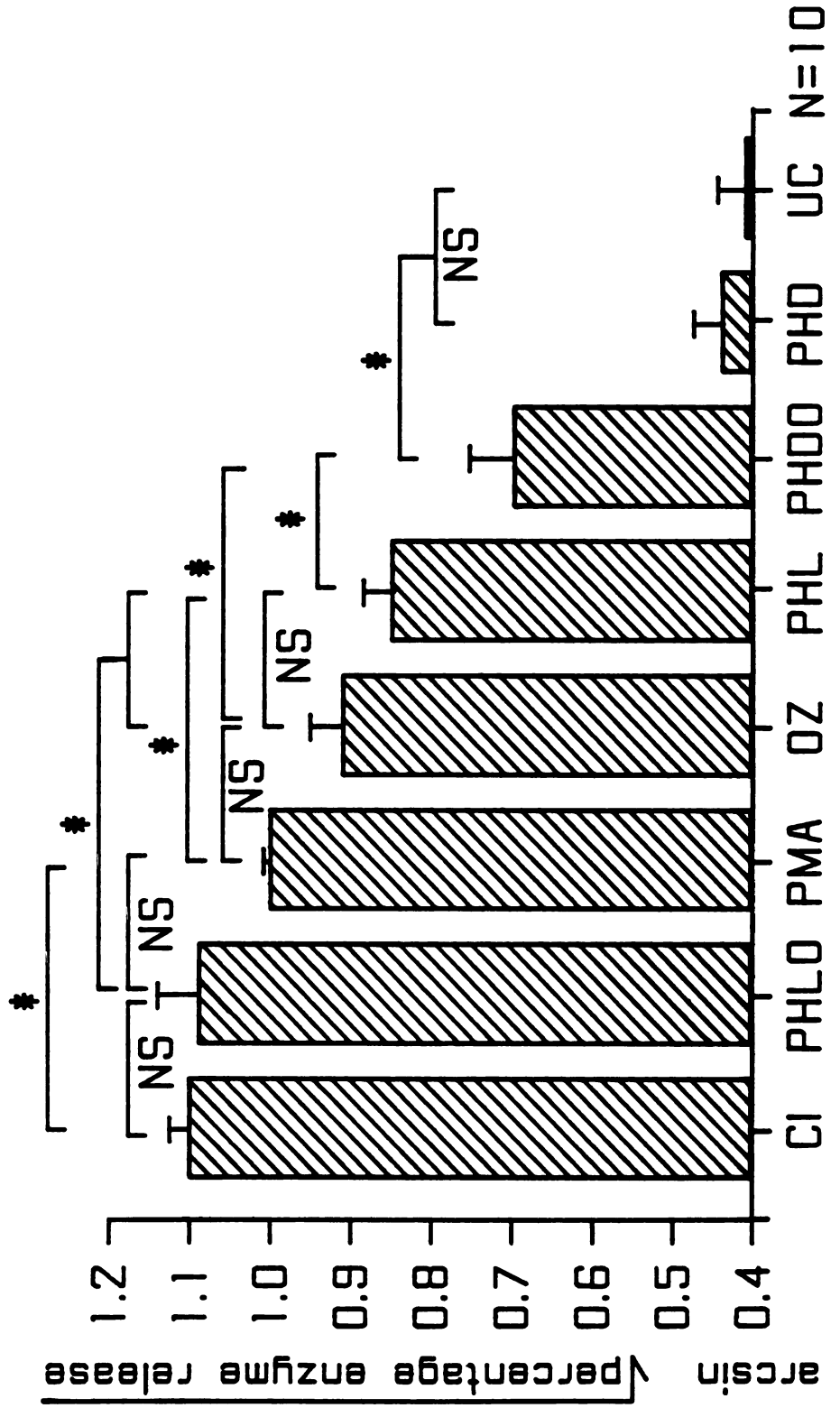

arcsin √percentage enzyme release
0.0
0.1
0.2
0.3
0.4
0.5
0.6
PHLO
PHL
CI
PMA
PHDO
PHD
OZ
UC
N=10
*
*
*
NS
NS

responses to PMA differing from the response to PHL. The responses induced by exposure of neutrophils to OZ and PHL were significantly greater than that to PHDO. The response to PHDO was also significantly different from the responses to PHD and of UC which induced no significant release of B12BP (Figure 3.6).

DISCUSSION: In the initial studies (enzyme release 1), calcium ionophore induced the highest release of BG but also produced the highest LDH release suggesting that the CI levels used (10^{-5} M) also induced either membrane damage or killed cells (Figure 3.1). It was not possible to conclude that CI induced a selective secretion of primary granules, although the data were suggestive. PMA induced virtually no BG or LDH release, suggesting that at the level of 200 ng the compound had no selective effects on neutrophils to cause the release of primary granules and that it caused little cell toxicity. It has been previously reported that PMA induces a selective release of specific granules in neutrophils.[34,53,54] Since the bovine neutrophil contains a majority of specific granules[99], major differences in BG release due to an agent which induces selective secretion of specific granules would have been unexpected. OZ stimulation also had no significant effect on primary granule release and was not toxic to cells.

Figure 3.6. Enzyme Release 4: Comparison of the BG, LDH, and B12BP Release by Bovine Neutrophils in Response to Stimulation by Soluble and Particulate Agonists and by *Pasteurella haemolytica*. Part III: B12BP Release.

Analyses of arcsin square root transformed data by OWA and LSD. Each bar represents the mean of B12BP released with a standard error bar. Abbreviations for treatment groups identical to previous figures. Horizontal brackets with a centered asterisk (*) enclose treatment groups that differ significantly. Horizontal brackets with a subtitle NS enclose groups with no significant differences. N-number of experiments.


arcsin √percentage enzyme release
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
CI
PHLO
PMA
OZ
PHL
PHOO
PHO
UC
N=10
NS
NS
NS
NS
NS
*
*
*
*
*
*

There was extreme variability in the results of BG assays, because of the extremely low levels of BG in bovine neutrophils. This required the use of four times the numbers of neutrophils (10 X 10^6) that are required in BG assays of human neutrophils.[54] It was necessary to incubate supernatants with substrate for 18 hours to generate quantifiable amounts of reaction product, whereas for other species, 2 to 4 hour incubations have been described. In contrast, the results of assays for LDH release had much less day-to-day variability. The total LDH released by the positive control cells (10 X 10^6) was always comparable to that of abnormal serum controls. There was excellent reproducibility between experiments (data not shown).

In the initial studies (enzyme release 2) of the neutrophil enzyme release in response to live and dead, opsonized and unopsonized *Pasteurella* organisms versus the unstimulated controls, the responses were incremental with the live opsonized, live, dead opsonized, and dead organisms inducing the most to the least responses, respectively, for both BG and LDH (Figure 3.2). These responses were similar to those previously reported in our studies of bovine neutrophil oxidative metabolism (Chapter 1) and are apparently related to the relative efficiency of neutrophil phagocytosis induced by the different treatments of the *Pasteurella* organisms. These data indicate a role for opsonins in the induction of enzyme release and affirms the

hypothesis that an undefined factor present in live organisms facilitates the uptake of organisms by neutrophils. The increases in LDH release induced by PHLO and PHL were as anticipated, since only viable bacteria are expected to damage or kill neutrophils. In concert with the results for LDH, a portion of the BG released by exposure to PHLO and PHL may have been due to cell lysis.

In studies (Enzyme Release 3) which compared the magnitude of BG and LDH responses caused by the agonists and *Pasteurella* organisms, CI again induced the highest release of both BG and LDH. There were virtually no (significant) differences in the quantities of BG released from neutrophils when induced by the *Pasteurella* groups, PMA, OZ, or in UC. For LDH, only PHLO and PHL caused significantly more LDH release than the remaining agonists (Figure 3.3). These data suggest that: 1) exposure of bovine neutrophils to *Pasteurella* organisms, even in the presence of opsonins, induces little selective primary granule (BG) release at an approximate cell to bacteria ratio of $\leq$ 25:1; 2) a portion of the BG released in response to PHLO and PHL is due to cell damage/lysis as shown by a concomitant elevation in LDH release; and 3) the release of primary granules should have a small or insignificant role in tissue damage attributed to the interaction of *Pasteurella* organisms with neutrophils.

In the studies (enzyme release 4) which compared the release of BG, B12BP, and LDH from neutrophils after stimulation by agonists and *Pasteurella* organisms, the responses were decidedly different and remarkably complex especially for B12BP. In this series of experiments, PHLO and PHL induced the highest release of BG and LDH exceeding the responses to CI. For BG, the quantities of enzyme released in response to PHLO stimulation were significantly greater than those to PHL, CI, and PHDO (Figure 3.4). For LDH, the quantities of enzyme released in response to PHLO, PHL, and CI were all significantly different from each other (Figure 3.5). The parallel responses in both of these assays again suggest that a portion of the BG release was via cell lysis and not entirely due to secretion. As in prior studies opsonization of the *Pasteurella* organisms, live or dead, markedly enhanced the enzyme release by neutrophils. Since the washing of organisms during their preparation should have removed most secreted products, such as endotoxin or cytotoxin we concluded that the LDH release was due to cell death and cell lysis induced by the ingested pathogens.

Calcium ionophore and PHLO stimulated the greatest release of specific granules from neutrophils, with the responses being nearly identical. The responses to CI were significantly higher than for PMA (Figure 4-6). This response was unexpected since PMA has been reported[54,55] to

induce a selective release of specific granules. Only the report by Gallin indicated similar responses to CI.[34] The propensity of CI to induce membrane damage (increased LDH release) may have also increased the quantities of B12BP released. A similar conclusion was reached for the neutrophil responses to live organisms (PHL) with the effect enhanced by opsonization (PHLO). Therefore, it was not possible to differentiate between the amounts of B12BP released due to neutrophil lysis and those due to secretion. In contrast to the previous data the release of B12BP stimulated by PMA appeared to be principally secretory, at the concentrations used (200 ng), since there was comparatively little LDH released.

In initial studies of enzyme release, the concentrations (particle/cell ratios) of particulate agonists used were the same as in the studies of neutrophil oxidative metabolism, and these concentrations were maintained, although cell numbers increased from 2 X 10^6 to 10 X 10^6. In the comparative studies of the three enzyme assays, extreme care was taken to assure that equal quantities of particulate agonists were provided for each 2 X 10^6 cells. Differences in the neutrophil responses induced by particulate agonists, e.g. *Pasteurella*, in initial and comparative response experiments may be partially explained by the presence of fewer particles per cell in the initial studies.

The differences in the responses to soluble agonists between initial and comparative protocols are less easily explained. In initial studies of oxidative metabolism, we found that by using the same concentrations of soluble agonists (PMA, CI) and doubling cell numbers there were virtually no differences in cell oxidative responses (data not shown). We postulated that the number of molecules of stimulant (agonist) per cell was not sufficient to induce maximal oxidative responses of all cells. In neutrophils and other cells, it is well known that the affinity states of receptors, and perhaps enzymes, may modulate cell responses, including enzyme release.

Regardless of the mechanisms of release, lysis or secretion, the quantities of specific granule components released in response to live opsonized, live, or dead opsonized *Pasteurella* organisms were substantial. Since the responses to OZ were also comparatively high, the presence of opsonized particles, live or dead, pathogenic or nonpathogenic, may facilitate the release of specific granules by bovine neutrophils. The high levels of specific granule enzymes in bovine neutrophils combined with a high rate of release or secretion in response to *Pasteurella* organisms could have a major role in the characteristic pulmonary lesions of pneumonic pasteurellosis.

Neutrophil specific granules have been characterized by Gallin[34], as "the fuse that ignites the inflammatory

process." Although primary granules are also released by bovine neutrophils in response to the *Pasteurella* organism, partially due to cell lysis, relatively low numbers (compared to specific granules) and relatively low release in response to the presence of the organisms seem to preclude significance in pneumonic pasteurellosis. Several investigators have implicated the release of primary granules with the modulation or "turn off" of the inflammatory response[34,35] and have disclaimed the role of primary granule components in tissue injury. The myeloperoxidase-halide pathway has been shown to facilitate the disruption of the NAD(P)H-oxidase enzyme complex leading to the cessation of superoxide production.[35] Primary granule products have also been shown to destroy alpha-1-antitrypsin, prostanoid metabolites, and C5a.[185,186]

CONCLUSIONS: In summary, bovine neutrophils respond to *Pasteurella haemolytica* by the secretion and/or release of primary and specific granules, a process enhanced by opsonins. A portion of the enzyme release of both granule subtypes is caused by the killing or lysis of cells induced by the ingested *Pasteurella* organisms. Because specific granules are present in greater numbers in bovine neutrophils[99] and have a greater propensity for secretion and/or release, regardless of mechanism, in response to the presence of *Pasteurella* organisms, we suggest that the

specific granules of bovine neutrophils have a major role in the pathogenesis of pneumonic pasteurellosis.

Future studies should involve the determination of the role of *in vitro* and *in vivo* blockers of the secretion of specific granules as a means of ameliorating pulmonary injury in pneumonic pasteurellosis. Since prior studies have shown that endotoxin facilitates the oxidative responses of bovine neutrophils, the effects of endotoxin on the release of neutrophil specific granules in combination with exposure to *Pasteurella* organisms should also be explored.

SUMMARY

My research into the influence of *Pasteurella haemolytica* and its products on the function of bovine neutrophils defined the *in vitro* oxidative responses of isolated bovine neutrophils by means of luminol-enhanced chemiluminescence (LECL) and the production of superoxide anion when stimulated by selected agonists and agonist concentrations. These responses were characterized by marked day-to-day variability within and between individual animals and were partially dependent upon the age of the experimental animals, and upon the mechanisms by which individual agonists activate neutrophils. Live *Pasteurella* organisms and/or opsonins enhanced the LECL and superoxide responses of stimulated neutrophils, although in comparative studies these responses were exceeded by non-biological entities.

In my endotoxin experiments, I confirmed that the presence of trace quantities of *Pasteurella haemolytica* (PH) or *Escherichia coli* (EC) endotoxins stimulated bovine neutrophils to produce increased quantities of superoxide anion in response to phorbol ester (PMA) and calcium ionophore (CI). These results imply that endotoxin exposure selectively enhances protein kinase C and Ca^{2+}-mediated pathways of neutrophil transduction, whereas there

were no differences the the pathways mediated by particulates [latex or opsonized zymosan (OZ)]. Diametrically, endotoxin exposure markedly decreased the LECL responses of neutrophils to PMA and CI, but again had no effect on the responses to particulate agonists. My experiments also determined that there was no species-specific effect of PH endotoxin.

My experiments defining the magnitude of B-glucuronidase from primary granules, vitamin B_{12}-binding protein from specific granules, and cytosolic lactate dehydrogenase secretion/release by stimulated bovine neutrophils represent, to my knowledge, the first studies of this kind in the bovine species. These experiments confirmed that the near selective release of specific granules in response to the *Pasteurella* organism was enhanced by the presence of live bacteria and/or opsonins. They confirmed that the organisms are capable of injuring neutrophils after they are phagocytosed, as indicated by the concomitant enhanced release of cytosolic LDH.

In summary, *Pasteurella haemolytica* induces bovine neutrophils to produce reactive oxygen species including superoxide anion, perhaps enhanced by low levels of its endotoxin, and facilitates a marked release of specific granules, processes enhanced by opsonic factors. All of these factors may have possible roles in the disease.

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