Effects of *Pasteurella haemolytica* on Pulmonary Vascular Adrenergic Mechanisms

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ABSTRACT

Pneumonic pasteurellosis is a significant disease in beef production medicine. The most recent information suggests that this disease is a $700 million dollar per year economic burden in bovine food animal production The medical and pathological characteristics of this disease are well documented. Many pathological findings associated with pneumonic pasteurellosis may be explained by disruption of the pulmonary vascular adrenergic system. However, only a limited amount of research has addressed the adrenergic system and its relationship to the etiology and pathophysiology of this disease. In an attempt to further investigate the contributions of the vascular adrenergic receptor mechanism to the development of pneumonic pasteurellosis a series of six experiments have been completed.

It is to be noted, that in 1999 the organism *Pasteurella haemolytica* was renamed *Mannheimia haemolytica*. The name change was based on the taxonomic features of the organism from other closely related organisms, in particular *Pasteurella multocida*.. The differences noted were identified and described by Dr. Mannheim in 1974. The familiarity of the past nomenclature and the lack of familiarity for the new nomenclature suggests that the more commonly recognized name of *Pasteurella haemolytica* should be used throughout this document.

Scientific evidence suggests that the disruption of the normal homeostatic mechanisms of the pulmonary vasculature to beta adrenergic agents may be part of the etiology of pneumonic pasteurellosis. The dynamics and kinetics of the involvement of the beta receptors, following prophylactic vaccination and in the disease state, has yet to be fully investigated with respect to the events associated with pneumonic pasteurellosis.

Evaluation of the time frame of the onset and duration of the events associated with the disruption of pulmonary vascular beta adrenergic receptor mechanisms revealed that an escalating

level of dysfunction occurs over the first 24-48 hour period after exposure to parenteral *Pasteurella haemolytica* and lasts for at least 21 days.

A component of*P.haemolytica* organism or contained in the vaccine using the organism is likely associated with the disruption of vascular beta adrenergic mechanism. This factor is, as yet, not specifically identified, however the likely culprit is the lipid A moiety of the endotoxin. Using the well defined and purified *Escherichia coli* endotoxin, trials were run to examine the effect of endotoxin on the pharmacological response of vascular associated beta adrenergic receptor mechanisms. The effects of *Escherichia coli* endotoxin, administered parenterally, on beta adrenergic receptor mechanisms were pharmacologically indistinguishable from those effects following parenterally administered *Pasteurella haemolytica*.

The nature of the disruption in the beta adrenergic receptor remains a mystery. The receptor mechanism involves at least two second messengers to initiate vascular relaxation. Initial activation of the beta adrenergic receptor with a beta selective drug starts a cascade of events involving adenylylate cyclase and cyclic adenylylate monophosphate (cAMP) and nitric oxide. A disruption in the receptor mechanism, as a result of the parenteral administration of *Pasteurella haemolytica*, which is "upstream" of adenylyl cyclase, would result in a diminished amount of cAMP when compared to the unvaccinated negative controls. An investigation of cAMP accumulation, at the receptor level was inconclusive.

The assessment of some previously used vaccines has demonstrated that there is an, as yet unidentified virulence factor, associated with these vaccines that results in the pharmacological disruption of beta adrenergic receptor mechanisms. Two newer vaccines, Once PMH® and One Shot® have been evaluated and there is evidence to suggest that these currently used vaccines also have the ability to disrupt beta adrenergic receptor mechanisms in rats.

The effects of parenteral *P. haemolytica* on the alpha-2 adrenergic receptor mechanism, is described. The alpha-2 receptor mechanism, unlike the beta receptor mechanism appears to increase the amount of vasoconstriction. The possibility that the alpha-2 adrenergic receptor could also mediate vasorelaxation under certain conditions was investigated. The evidence suggests that in the presence of high alpha-1 mediated vascular tone, the alpha -2 receptor can cause vasorelaxation. Evidence, from other scientists active in this area of investigation, suggests that a vasorelaxation response may be mediated by nitric oxide. Elimination of the nitric oxide mediated relaxation may offer an explanation for the increased vasoconstriction noted with alpha-2 selective drugs after exposure to parenteral *P. haemolytica*.

Finally, the importance of the beta adrenergic receptor to the disease process is addressed by elucidation of one of the mechanisms by which Micotil 300® (tilmicosin phosphate) acts to improve cattle with symptomatic pneumonic pasteurellosis. The rapid improvement of animals on Micotil 300®, with-in 24 hours suggests that there is a mechanism beyond the antimicrobial effect of the drug that mediates the clinical improvement. Evaluation of the effect of Micotil 300® demonstrates a pharmacologically measurable amount of beta adrenergic activity with respect to the bovine pulmonary artery and vein.

Based on the conclusions drawn as a result of these experiments, the adrenergic system in general, and the beta adrenergic system in particular are important to the development of pneumonic pasteurellosis in cattle. The beta adrenergic system is affected by endotoxin. Further, these receptors maybe responsible for the mediation of the pathological and clinical signs associated with pneumonic pasteurellosis.

In conclusion, these investigations have suggested, that it is likely that a disruption in the homeostatic mechanisms mediated by the beta and alpha-2 adrenergic receptors are intimately involved in the development of post vaccination receptor failure as well as the pathophysiology associated with pneumonic pasteurellosis in cattle.

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LIST OF ABBREVIATIONS

a-1	alpha-1 adrenergic receptor
a-2	alpha-2 adrenergic receptor
ANOVA	analysis of variance
APC	antigen presenting cell
ARDS	acute respiratory distress syndrome
ATP	adenosine triphosphate
BAR	beta adrenergic receptor
BARC	beta adrenergic receptor complex
BAL	broncho-alveolar lavage
BALT	broncho-alveolar associated lymphatic tissue
BHI	brain heart infusion broth
BRDC	bovine respiratory disease complex
BRSV	bovine respiratory syncitial virus
BSA	bovine serum albumin
BVD	bovine viral diarrhea
Ca^{++}	calcium
C_5A	complement component
C_3A	complement component
cAMP	cyclic adenosine monophosphate
cAPK	cAMP dependent protein kinase
cGMP	cyclic guanosine monophosphate
CFU	colony forming units
DAG	diacylglycerol
ECAM	endothelial cell adhesion molecule
EC_{50} / ED_{50}	effective concentration or effective dose for 50% of subjects
EC	*Escherichia coli*

EDRF	endothelial derived relaxation factor
EU	endotoxin unit
G-a	alpha protein of the G-protein receptor mechanism
G-b	beta protein of the G-protein receptor mechanism
G-g	gamma protein of the G-protein receptor mechanism
GDP	guanosine diphosphate
GTP	guanosine triphosphate
ICAM	intracellular adhesion molecule
Ig-A	immunoglobulin -A
Ig-E	immunoglobulin-E
Ig-G	immunoglobulin -G
IL-1	interleukin-1
IL-6	interleukin-6
IP_3	inositol triphosphate
KDO	2-keto-3-deoxycytulosonic acid
L-NAME	N^w-nitro-L-arginine methyl ester
LPS	lipopolysaccharide, endotoxin
MAC	membrane attack complex
MHC	major histocompatability complex
MLCK	myosin light chain kinase
MODS	multiple organ dysfunction syndrome
NANC	nonadrenergic noncholinergic
NFkB	nuclear factor kappa B
NO	nitric oxide
NOS	nitric oxide synthetase
PAF	platelet activating factor

PAM	pulmonary alveolar macrophages
PH	*Pasteurella haemolytica, (Mannheimia haemolytica)*
PI-3	parainfluenza 3 virus
PLC	phospholipase C
PMN	polymorphonuclear, neutrophil
ScEM	scanning electron micrographs
SEM	standard error of the mean
TCR	T cell receptor
TF	tissue factor
TNF-alpha	Tumor Necrosis Factor alpha
VCAM	vascular cell adhesion molecule
VIP	vasoactive intestinal peptide

INTRODUCTION

Bovine Respiratory Disease Complex and more specifically pneumonic pasteurellosis (also known as shipping fever or transit fever) has been recognized in cattle occurring as a unique series of events that are described by a complex, multifactorial equation that as yet, is not completely understood. The disease pneumonic pasteurellosis is defined by the presence of *Pasteurella haemolytica* (PH) in the lower respiratory tract simultaneously with the diagnosis of fibrinopurulent broncho-pneumonia. Exposure of the host to *Pasteurella haemolytica* is not sufficient to induce the disease. Further, the bacterium *Pasteurella haemolytica* A-1(PH), is a commensal found on the mucosal surface of the nasal and pharyngeal areas of most healthy and susceptible ruminant species. Many investigators have demonstrated that the action of respiratory viruses (Infectious Bovine Rhinotracheitis (IBR), Parainfluenza-3 (PI-3), Bovine Viral Diarrhea Virus (BVD), and Bovine Syncytial Respiratory Virus (BSRV)) act synergistically with PH to initiate pneumonic pasteurellosis. The viral component appears to disrupt the physical, cytological and immunological pulmonary defense mechanisms rendering the host more susceptible to bacterial invasion. Other factors such as physical stressors including; vaccination, castration, crowding, starvation, dehydration, cold exposure, and transportation also enhance the likelihood of pneumonia in cattle. Stressors that act to increase circulating cortisol can result in a decrease of pulmonary defenses.

Previous studies, completed in this laboratory, demonstrated that live *Pasteurella haemolytica* A-1 (approximately 10^5 colony forming units) injected intraperitoneally causes both beta and alpha-2 adrenergic receptor dysfunction in rats. Similar results have been described for the pulmonary vasculature in sheep. Disruption of vascular associated adrenergic mechanisms appears to be a component in the molecular pathogenesis of pneumonic pasteurellosis.

Lipopolysaccharide (LPS) or endotoxin, of *Pasteurella haemolytica* A-1 may be the active bacterial cellular component that causes adrenergic dysfunction. Beta adrenergic receptors are integral to many metabolic and physiological functions including: vascular smooth muscle relaxation, inhibition of the leukocyte chemotaxis, modification of fluid extravasation in the lung, and modulation of coagulation events. One of the mechanisms by which these events are likely to be induced is through a change in the relative concentrations of the two intracellular second messengers, cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP). PH endotoxin has been demonstrated to disrupt the cellular concentrations of cAMP and cGMP.

II. REVIEW OF RELATED LITERATURE

A) Overview of the Problem

The Bovine Respiratory Disease Complex (BRDC) is a group of undifferentiated diseases of young cattle. This group of diseases is characterized by signs and symptoms such as nasal discharge, coughing, fever, lethargy and non-specific lung sounds (crackles and wheezes) in the cranial and ventral lung fields. Pneumonic pasteurellosis is characterized by the isolation of PH as the predominant bacterial agent involved with a fibrinous bronchopneumonia in cattle (Blood 1988).

Bovine Respiratory Disease Complex has been a significant problem for years in feedlot yearlings (Jensen 1976A; Jensen 1976B) and in cow-calf operations (Salman 1991B) in the United States and Canada. Recent estimates of calf mortality due to respiratory infections have been given to be 7.6%, and represents 9.8% of costs of all diseases monitored (Wittum 1993).

The high cost is, in part, due to the greater age-increased dollars invested per calf at the time of illness or death (Salman 1991A; Salman 1991C). Data indicate that the incidence of BRDC is greater in calves (cattle under 500 lbs) than in adults and surprisingly greater among dairy calves than beef calves (USDA 1992; Dargatz 1996) . Total lost revenue, due to death from bovine respiratory disease, to American producers was approximately $100 million dollars in 1986 (Wohlgemuth 1987). More recently the United States Department of Agriculture, Center for Epidemiology and Animal Health (USDA-CEAH) estimates the cost of BRDC to the United States to be between $400-500 million dollars per year (Dargatz 1996).

Prevention of pneumonic pasteurellosis has been based on improving preconditioning methods, herd management practices (Wilson 1985; Loan 1992; Smith 1992; Radostits 1994) and the use of prophylactic vaccination (Wilson 1985; Smith 1988; Shewen 1988A; AVMA Council Report 1993). Preconditioning is directed at decreasing stress, increasing the plane of nutrition and

improving weight gain, feed efficiency and disease resistance (Wilson 1985; Loan 1992; Smith 1992; Radostits 1994).

The efficiency of vaccines in reducing morbidity and mortality as a consequence of pneumonic pasteurellosis among food animals is controversial. Vaccines are thought to act by enhancing immunologically based disease resistance in the medium to long term period (months to years) without having to expose susceptible individuals to the disease itself.

Recent studies from this laboratory suggested that parenteral administration of *Pasteurella haemolytica* A-1 may initiate subclinical changes in the pulmonary vasculature of calves (Weekley 1993A; 1993D), sheep (Weekley 1991A; 1991B) and the aorta of rats (Weekley 1993B). These subtle pathophysiological changes appear to increase disease susceptibility and incidence in the immediate post-vaccination period.

A positive economic and disease reducing effect of vaccines for pneumonic pasteurellosis, have been reported (Smith 1988; Shewen 1988B; Cravens 1993; Cravens 1993A). Other scientists, using epidemiological data, presented evidence that vaccines for pneumonic pasteurellosis may not be as effective as previously thought (Martin 1983; Thorlarkson 1990). These findings may be due to a period of potential post-vaccination impairment that may occur, simultaneously in time, with a high stress environment. Any vaccine related physiological /vascular impairment may be deleterious to normal pulmonary physiological and anatomical defenses so when combined with the immunodepressant effects of stress could result in increased morbidity and mortality (Weekley 1993B) . In the scientific literature, which considers prophylactic vaccination an effective tool, the immediate post-vaccination intervals were stress free (Smith 1988; Shewen 1988B; Cravens 1993; Cravens 1993A). This stress-free environment may be very different than that found in actual practice where the producer may gather the animals, vaccinate, castrate, implant and then immediately transport to the feedlots (Dyer 1982; Shoo 1989).

Vaccine efficacy is dependent on many factors. The PH bacteria are present as normal nasal pharyngeal flora. Given their close anatomical proximity to the lung, they are potentially able to infect any animal that is immunocompromised before the establishment of the protective immunity of the prophylactic vaccination (10-21 days) (Evermann 1988; Tizard 2000). Therefore, this results in an increase in disease susceptibility due to a vaccine induced, stress mediated, compromised homeostasis, that has been reported as a vaccine failure (Martin 1983). It is likely that the interaction of stress and disruption of the beta adrenergic receptors, during the PH vaccine post-exposure period, is the cause of the increases in morbidity and decreased weight gain noted by Thorlarkson (Thorlarkson 1990) and increases in mortality noted by Martin (Martin 1980; Martin 1983).

Previous work completed in this laboratory reported that exposure to PH modifies the beta adrenergic receptor (BAR) response of the pulmonary vasculature to vasoactive drugs. Altered adrenergic vasoreactivity could possibly contribute to the development of pulmonary congestion and inflammation associated with increased susceptibility to pneumonic pasteurellosis. Parenteral PH also appears to cause a disruption in the integrity of the pulmonary endothelium in sheep and calves (Weekley 1991; 1991A; 1991B; 1993A; 1993D) and the aortic endothelium in rats (Weekley 1993B). These results suggest that exposure to PH may induce conditions within the pulmonary and systemic vasculature that increase the risk of pulmonary disease in the short term.

B) *Pasteurella haemolytica*

Pasteurellacae are gram negative, non-motile coccobacilli or rods. These bacteria are facultative anaerobes and are fermentative. *Pasteurellacae* may be divided into several species including: *P. haemolytica, P. multocida*, *P. pneumotropica*, and many others that are relatively host species specific (Mutters 1989). PH are animal pathogens primarily of the ruminant respiratory system however, infections of man, rodents, dogs and lagomorphs have been reported. *Pasteurella*

haemolytica have been found to be part of the normal nasopharyngeal flora, however these organisms are rarely found in significant numbers in the lungs of healthy unstressed calves (Corbeil 1985; Shewen 1993) .

A proposal in the recent scientific literature suggested that *Pasteurella haemolytica* be renamed as *Mannheimia haemolytica* (Angen 1999). This proposal is put forward based on a re-evaluation of the taxonomic dissimilarities between *Pasteurella haemolyica* and *Pasteurella multocida*. Thismay be valid, however, most veterinary clinicians and researchers are most familiar and currently using the original nomenclature. Though the Angen (1999) paper is accepted at this time I will complete this dissertation using the more recognized clinical terminology of *Pasteurella haemolytica*.

Pasteurella haemolytica A1 is the primary bacterial agent isolated in pneumonic pasteurellosis however, infections leading to fibrinous bronchopneumonia can also result from *Pasteurella multocida* (Dungworth 1983; Wiksie 1985). *Pasteurella haemolytica* is easily identified as a smooth round, grayish to off-white colony that initiates beta-hemolysis on a Colombia blood agar plate made containing 5% sheep blood. This same organism fails to grow on McConkey's medium (Joklik 1993; Shewen 1993). PH falls into one of two distinct biotypes based on its ability to ferment different sugars. Biotype A has the ability to ferment the sugar arabinose while biotype T has the ability to ferment trehalose. Further classification is based on serotype. Serotype is defined by soluble or extractable capsular surface antigens using a passive haemagglutination procedure or rapid plate agglutination test. Sixteen serotypes are recognized currently and are designated by the numbers 1 through 16. Those serotypes numbered 3, 4, 10, 15 are all trehalose fermenters, biotype T. The remaining serotypes are arabinose fermenters, biotype A. Some PH cannot be adequately serotyped to be identified within the current classification scheme (Joklik 1993; Shewen 1993). The most common isolate found associated with pneumonic

pasteurellosis is biotype A serotype 1 in cattle and biotype A serotype 2 in sheep (Dungworth 1983; Wiksie 1985).

Four virulence factors have been described for PH. These include: fimbriae, polysaccharide capsule, leukotoxin, and endotoxin (lipopolysaccharide). Fimbriae are capsular protein appendages serving as adhesins that interact with specific receptors on selective eukaryotic cells. It is thought that fimbriae enhance adherence of the organism to the respiratory mucosa (Confer 1990). Two types of fimbriae are recognized to be associated with PH biotype A, serotype 1. One type are large rigid (12nm in length) and narrower (5nm) are more flexible fimbria (Morck 1987) . Though present in all growing colonies this factor is labile in its' presence. It has been demonstrated that shaking a viable culture during the log growth phase can inhibit the formation of fibriae (Confer 1990).

A second major virulence factor associated with PH is capsular polysaccharide or the glycocalyx. Glycocalyx associated with PH grown in-vivo is greater in thickness than that grown in-vitro (Morck 1989). Capsular polysaccharide appears to also vary with the different serotypes and may be a major antigenic factor in the immunological protection induced by prophylactic vaccination. This was not explained in a series of experiments. The glycocalyx is a complex polysaccharide that enhances the attachment of PH to the alveolar luminal membrane and bronchial epithelial surfaces (Morck 1987; Morck 1989; Confer 1990). Capsular polysaccharide appears to increase neutrophil migration though it decreased the PMN's ability to kill PH A1 (Chae 1990). Further, encapsulated PH were less susceptible to serum agglutination, complement-mediated killing (MAC attack), and phagocytosis by neutrophils (Confer 1990). The capsular portion of PH may also be involved in the pathogenesis of pneumonic pasteurellosis and the resulting mortality by inducing anaphylaxis in cattle (Rice Conlon 1993). Again, this factor was not investigated further within the research performed for this dissertation.

The third major virulence factor of PH-A1 is the leukotoxin. Leukotoxin is a high molecular weight multiprotein complex, secreted from the bacterial cell and is toxic to both ruminant leukocytes and platelets (Shewen 1982; Clinkenbeard 1989A; Confer 1990; Clinkenbeard 1991; Shewen 1993). Leukotoxin appears to be elaborated by all strains of PH (Clinkenbeard 1989A). This is a heat labile molecule that, in low doses, appears to inhibit neutrophil mediated phagocytosis, oxygen free radical production and leukocyte proliferation. At higher concentrations there is cell death (ruminant leukocytes and platelets) by lysis (Shewen 1982; Clinkenbeard 1989A; Confer 1990; Clinkenbeard 1991; Shewen 1993). Lysis occurs secondarily to the formation of transmembrane pores that result in the disruption of the potassium, sodium, calcium and water homeostatic equilibration across the leukocyte cell membrane. This can occur within several minutes of the exposure of leukocytes to the toxin and ceases, due to decreased elaboration, within 60 minutes. It has been shown that Ca^{++} is required for lysis of neutrophils (Confer 1990). The subsequent release of neutrophil lytic enzymes and oxygen free radicals are both tissue damaging and chemotactic for other leukocytes increasing the likelyhood of autogenous tissue damage (Confer 1990). Platelet destruction due to the leukotoxin can lead to the activation of the coagulation system through the elaboration of fibrinogen resulting in the generation of thrombin, perivascular and extra-vascular fibrin leakage (Confer 1990; Clinkenbeard 1991). However, unlike endotoxin, leukotoxin appears to have no deleterious effect on the vascular endothelial cells (Clinkenbeard 1992). Within the pulmonary alveolar spaces and vasculature, leukotoxin reduces cellular defenses, neutrophils and alveolar macrophages. Also the generation of thrombi is secondary to the leukotoxin induction of the intravascular coagulation system and the destruction of platelets (Confer 1990).

LPS or endotoxin, the fourth major virulence factor, is a complex amphiphilic moiety, derived from the wall of gram negative bacteria. Endotoxin is a heat stable, potentially lethal toxin released on the destruction of a gram negative bacteria (Confer 1990). There are many reasons to consider LPS as the spearhead in the pathogenesis of pneumonic pasteurellosis. LPS specific

antibodies appear to enhance opsonization of bacteria in a mouse model of pasteurellosis (Wilson 1992) . Unfortunately, these antibodies are insufficient in-vivo to protect the animal from the disease (Confer 1986). Secondly, LPS has been demonstrated to damage bovine endothelial cells in-vitro (Paulsen 1989; Breider 1990; Breider 1991). There is some evidence to suggest that the LPS from PH may modify its virulence properties in-vitro when comparing nasopharyngeal acquired bacteria versus the pathogenic bacteria found in the lower respiratory tract (Lacroix 1993). Studies with purified endotoxin have demonstrated that intravenous administration in calves can result in the release of thromboxane A_2 , prostaglandins, serotonin, cAMP and cGMP (Emau 1987). The pathogenic effects of LPS are to some degree, dose dependent . At low concentrations of endotoxins, phagocytosis is inhibited while at higher concentrations phagocytosis is increased (Confer 1986). Some of the local pulmonary affects of LPS in sheep administered parenterally (IV) include; pulmonary inflammation, hyperemia, hemorrhage, edema and congestion (Brogen 1989). LPS can also lead to increased neutrophil adherence to the vascular endothelium as well as increased arachidonic acid release indicating endothelial cell activation. Activation of the neutrophil has been associated with an increased severity of lesions in pneumonic pasteurellosis (Slocombe 1982; Breider 1988). The modified neutrophil response, in the presence of LPS, can increase the release of endogenous vasoactive mediators, the generation and release of oxygen free radicals and proteolytic enzymes. Response to endotoxin is dependent on several factors including: animal species, dose, route and rate of administration, and ease of absorption into the circulatory system (Confer 1990). Some of the changes in the hematological and pulmonary system, in response to endotoxin, are mediated by immune protective cells and their elaboration of the inflammatory cytokines: interleukin 1(IL-1), interleukin 6 (IL-6) and Tumor Necrosis Factor-alpha (TNF-alpha). Cytokines are associated with an acute endogenous phase response leading to inflammation (Emau 1987; Confer 1990; Joklik 1993). The o-polysaccharide chain of LPS has been suspected of reducing the number of airway beta adrenergic receptors (BAR) in the guinea pig lung (Schreurs 1982; Schreurs 1983).

Lipopolysacccharides have three basic structural components, lipid A moiety, the core region (containing the inner core and outer core) and an o-specific polysaccharide chain that is unique to each organism (see figure #1).

The lipid A moiety is the major, pharmacologically active, part of the lipopolysaccharide (Raetz 1990). The lipid A portion activates host defenses, inducing the clinically recognizable septic signs of sepsis including: hypotension, decompensating cardiac function. Further the lipid A moiety acts as a pyrogen resulting in fever and a mediator of lung and vascular damage (Lacroix 1993). The lipid A portion of LPS is highly conserved among eubacteria, suggesting its importance to the survival of these organisms. The hydroxyl group (C6) serves as the attachment site of 2-keto-3-deoxyocytulosonic acid (KDO). There are two fatty acid chains attached to the glucosamine disaccharide which result in the hydrophobic properties of the lipid A molecule. The number of fatty acid substituents vary for the numerous bacterial species and even for bacteria grown in single culture. These variations in structure may alter toxicity without altering other types of biological activity (Lacroix 1993). Potency has been demonstrated to increase with up to six fatty acid substitutions. However, removal of all fatty acids results in a loss of toxicity. Further, the removal of either the C1 or C4 phosphate groups can also result in loss of toxic activity (Joklik 1993; Lacroix 1993).

The core region consists of an inner and outer region. The inner core region is bound to the disaccharide of the pharmacologically active lipid A molecule by the molecule KDO. KDO and the core structure may be conserved within the organism, although this molecule differs among bacterial families. Those bacteria lacking the KDO segment of the LPS molecule are non-viable (Joklik 1993).

The O-specific polysaccharide, a side chain sugar, is responsible for the serotype antigenic determinants in PH (Chester 1973; Lacroix 1993). Serotypes 2 and 8 lack the o-specific

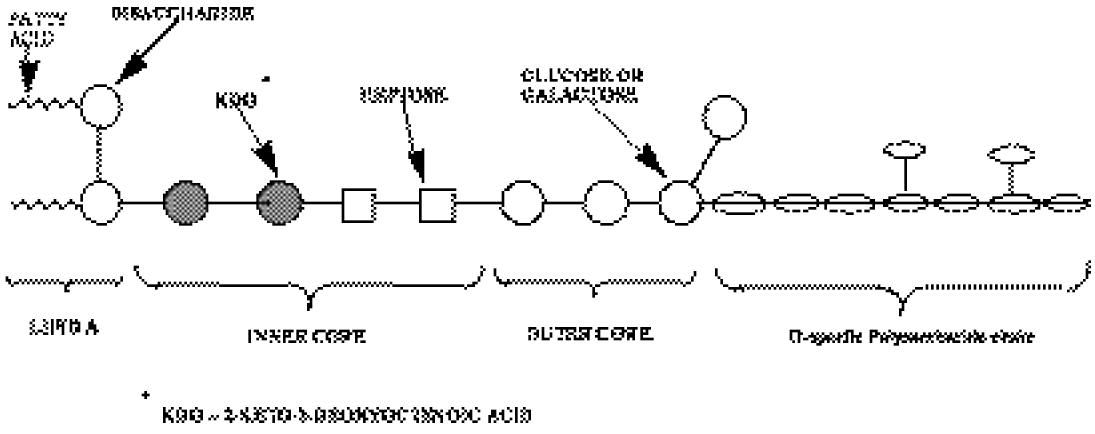


FIGURE # 1- Schematic representation of endotoxin or lipopolysaccharide

polysaccharide side chain in their endotoxin and are considered to have a rough colony morphology. Other serotypes (including A-1) are considered to have a smooth colony morphology (Leuderitz 1966; Goldman 1980; Rimsay 1981; Emau 1987; Shewen 1993). Bacteria of rough colonies have approximately 1.5% endotoxin and smooth cells 8% endotoxin by dried cell weight (Emau 1987). There also appears to be differences in the pathogenicity of the rough and smooth types of PH. Increases in cAMP were more prolonged, in-vitro, for smooth LPS than for rough LPS (Emau 1987). There are also differences in the induction of the complement system with respect to the actions of LPS (Emau 1987). The lipid A moiety can induce the classical complement pathway while the O-specific polysaccharide chain activates the alternative pathway of the complement system (Vukajlovitch 1987). Despite the integral nature of endotoxin to the pathogenicity of the organism, elevated antibody titers to LPS does not correlate with the resistance to pneumonic pasteurellosis in cattle (Confer 1986; Confer 1989).

In their review article of the relationship of endotoxin and the development of disease, Morrison and Ryan suggest that the toxicity of endotoxin is not solely related to its direct pharmacological effect on the host but also on its induction of endogenous tissue factors that may have a profound effect in pathogenesis (Morrison 1987). Specifically, endotoxin has been

demonstrated to induce the complement anaphylatoxins (C3a, C5a) (Vukajlovitch 1987), Arachidonic Acid metabolism (Hagmann 1985; Aderem 1986), Tissue Factor activity (Morrison 1978), Platelet Activating Factor (Doebber 1985) the cytokines Interleukin 1 (Bayne 1986) and Tumor Necrosis Factor-alpha (Beutler 1985; Beutler 1985) .

C) Lungs and the Pulmonary Vasculature

Some unique and species specific features associated with both the bovine pulmonary physiology and anatomy may significant in of the etiology of shipping fever. There is a common clinical perception that cattle are at an increased risk for pulmonary infection and disease due to PH.

Bovine pulmonary anatomy and physiology are significantly different than those of man and other mammals (Veit 1978; Green 1982; Robinson 1984; Lekeux 1993; Weekley 1995; Wren 1995). In the bovine, the right thoracic cavity encompasses four lung lobes (cranial, middle, accessory and caudal) while the left thoracic cavity has two lung lobes (cranial, caudal) (Robinson 1984; Weekley 1995; Wren 1995). The volume of the bovine lung is small relative to the size and weight of the animal (Robinson 1984; Weekley 1995; Wren 1995) and there is a lower functional capacity as compared to other mammals (Tenney 1963; Veit 1978; Robinson 1984; Weekley 1995; Wren 1995) . The functional capacity is greater in the fully grown adult than the calf. In the calf up to 42% of the minute-ventilation is dead space (Slocombe 1982) whereas in the young adult (10.5 months) the dead space ventilation has increased to 68% of the minute ventilation (Hales 1968; Slocombe 1982).

Pulmonary airway anatomy consists of two clinically different divisions, the upper respiratory tract and the lower respiratory tract (Liggitt 1985). The upper respiratory tract consists of the oro-pharynx, larynx, trachea and mainstem bronchi. The lower respiratory tract starts below the mainstem bronchi. The trachea and main stem bronchi divide into successive, sequentially

smaller generations. The first generation starts just distal to the cricoid cartilage of the larynx and each subsequent bifurcation represents another generation. Each bronchus is paralleled by an artery, vein and nerve numbered for their generation. In general, in mammals, there are approximately ten to twelve generations of bronchial divisions (this is variable between individuals) that exist from the trachea to the respiratory bronchioles (Breeze 1985). Supporting cartilage exists in the conducting airways down to the level of the terminal bronchioles (approximately 1-2 mm in diameter or 8-10 generations of bronchial bifurcations). At this level, the cartilage diminishes and tends to form discrete plates that may move dynamically in response to the airway vascular smooth muscle. Airway smooth muscle is a prominent anatomical and functional feature of the terminal and respiratory bronchioles in both man and ruminants (Green 1982; Breeze 1985). This airway smooth muscle diminishes to the level of the alveoli where it is completely absent. Bronchial smooth muscle tone is under the control of both the autonomic nervous system and systemic endogenous hormones (Breeze 1985).

In most species, lobar bronchioles arise as part of the complex of bronchioles that is formed by the termination of the trachea, called the carina. The bovine lung has an additional bronchial bifurcation (a lobar bronchus) to the right cranial lobe which arises directly from the trachea, crainial to the carina (Getty 1975; Veit 1978; Schummer 1979; Robinson 1984; Wren 1995) . Although this may seem to be an innocuous anatomical deviation, direct gravitationally dependent drainage of tracheo-bronchial secretions into the right cranial lobe may potentially "seed" acini with bacteria and / or occlude bronchioles with protective mucoid polysaccharides associated with the mucociliary escalator (described below under lung physical defenses).

The bovine lung has complete interlobular septa and a dearth of inter-alveolar pores, also called pores of Kohn (Veit 1978; Robinson 1984; Weekley 1995; Wren 1995). This results in poor collateral communication among the secondary lobules and acini with an increased likelihood that a single mucoid thrombus could occlude an entire primary lobule (Robinson 1984; Wren

1995). Occlusion of an alveolus or terminal bronchiole can result in a ventilation perfusion mismatch which would cause a local (at the level of the acinus) anaerobic environment. Pulmonary vascular constriction occurs as a response to alveolar hypoxemia. This pre-capillary vasoconstriction can cause a functional and physiological shift of blood away from the occluded alveolus and potentially increases right heart work over time (Veit 1978; Green 1982; Robinson 1984; Breeze 1985; Barnes 1995). The resultant shift in blood away from the alveolus also diminishes the likelihood of the diapedesis of neutrophils and increases the anaerobic nature of the acinus.

Dynamic lung compliance is lowered in the normal healthy calf more than in other young mammals (Lekeux 1993). Dynamic lung compliance is the ability of the lung to affect a volume change to a given intrathoracic pressure change. A decrease in compliance may occur as a result of terminal smooth muscle mediated bronchial closure or bronchial obstruction (Lekeux 1993). Decreased dynamic compliance is an indication of early obstructive airway disease (Geunter 1982; Green 1982). Thus, the calf must exert more effort (with respect to intrathoracic negative pressure) for normal inspiration and is therefore more susceptible to vascular blockage secondary to airway emboli (Veit 1978; Green 1982; Robinson 1984).

The vascular, lymphatic and neural elements parallel each other along the bronchial tree structure. The lung has two arterial systems functioning within the pulmonary tissue; the first system is the tissue nutritional blood supply and the second is a functional supply. The nutrient supply, the bronchial artery, arises from the thoracic aorta to supply oxygen to the pulmonary tissues. The second or functional blood supply arises from the right ventricle to enter the lungs as the pulmonary arteries. The arteriol vascular system then branch in concert with the bronchial airways to eventually form the pre-capillary arterioles, a capillary net and then post-capillary venules at the level of the alveolus. The functional supply enters the alveolar blood-gaseous exchange area through the fine capillary bed vessels that are in intimate contact with the respiratory alveolar

membrane of the terminal respiratory tract. The barrier between the vascular elements and the airway elements consists of a double basement membrane with a single layer of airway epithelial cells and a vascular layer of luminal endothelial cells (Green 1982; Lekeux 1993).

Bovine pulmonary arterial vessels have a significantly greater tunica muscularis than other mammals (Tucker 1975; Robinson 1984; Weekley 1995; Wren 1995). Pulmonary vascular reactivity to hypoxia is therefore proportionately greater (Robinson 1984; Wadsworth 1994; Weekley 1995) as is the ability of the bovine lung to correct a ventilation perfusion mismatch (Veit 1978; Robinson 1984; Weekley 1995). There is a significant vascular collateral circulation at the level of the pre-capillary and capillary bed due to the anastomosis of the bronchial arterioles with the pre-capillary arterioles in the bovine (Schummer 1979). Overall these unique anatomical features of the bovine pulmonary vasculature results in dramatic fluctuations in perfusion / ventilation ratio and contribute in part to the pathogenesis of pneumonic pasteurellosis.

The pulmonary capillary vasculature also serves as a "vascular filter" for emboli, thrombi and the cellular elements of the vascular system. Those thrombi filtered by the lungs are removed by phagocytosis. This is primarily by circulating monocytes and vascular endothelial cells. Beyond the pulmonary actions, systemic and splenic vascular sequestration of lymphocytes, granulocytes, platelets and macrophages comprise the physiological and hemostatic attempts to regulate circulating vascular elements (Irwin 1975; Breeze 1985).

The pulmonary endothelium is a complete, non-fenestrated, single layer, squamous cell sheet lining the vasculature. Adjacent cells have tight cell junctions with physiologically active intracellular pores approximately 4 nm in diameter. Ordinarily these cells are impervious to macromolecules though there is bi-directional transport of ions and macro-molecules (Ryan 1982; Breeze 1985). The endothelium is a metabolically active site (Ryan 1982). The endothelial cells have surface caveolae and form pinocytic and phagocytic vessels. Many vasoactive mediators and

hormones are removed from the circulation by the endothelium including, serotonin, nor-epinephrine, prostaglandins (PGE, PGF) and thromboxane (Breeze 1985). As well, pro-coagulatory molecules such as ADP are inactivated by endothelial cells (Ryan 1982). As a metabolic site, the endothelium elaborates PGI-2, PGE-2, thromboxane and activates angiotensin-1, angiotensin-2 (Ryan 1982; Breeze 1985). Under normal conditions the endothelium is actively non-thrombogenic in nature (Ryan 1982). However, when damaged the endothelium can activate the both the coagulation and complement systems (Ryan 1982).

In summary the combination of the small alveolar surface area (Veit 1978), the lack of alveolar communication (pores of Kohn), and the amplification of the vasoconstriction response due to an increased pulmonary vascular muscular tunica may also contribute to the development of both an anaerobic environment and a local metabolic acidosis. The possible effect of this combination of circumstances may be a suppression of bacterial clearance (Thompson 1974) and suggests the possibility of an alveolar environmental change from an aerobic to an anaerobic environment. This may result in a changes in the relative pathogenicity of *P. haemolytica*. Further, it has been demonstrated that anaerobically challenged macrophages loose their ability to phagocytose bacteria but retain their ability to generate and release free radicals after cell degeneration (Matthews 1987; Cazin 1990; Thompson 1992). The simultaneous association of the above described multiple factors may result in both pulmonary tissue damage as well as a compromise of the lung's physical antimicrobial defenses in the presence of an increased PH endotoxin challenge (Wessman 1964).

D) Lung Defense Mechanisms

Lung defenses may be divided into three general categories: physical defenses, immunological defenses and cellular defenses. It is thought that beyond the second generation of bronchioles the environment is sterile and maintained so by the cellular and immune defenses (Liggitt 1985).

The upper respiratory tract is protected by physical and immunological defenses (Veit 1978; Robinson 1984; Breeze 1985; Liggitt 1985). The nares are the external entrance to a series of blind and connecting passages called conchae or turbinates (Schummer 1979). These passages are lined by a mucus secreting epithelium that traps particles in the air turbulence caused by inhalation. This is termed turbulent filtration (Liggitt 1985) and is effective in removing particles greater than 10 μm (Dungworth 1983; Reece 1991). Trapped particles are then carried out of the turbinates on a mucocilliary blanket and either swallowed or eliminated in a mucoid secretion from the nares (Liggitt 1985; Reece 1991). A second physical defense of the upper respiratory tract is the mucociliary escalator. This is a thin layer (approximately 5 mm thick) of mucus carried on cilia that extend from the terminal bronchioles to the level of the larynx. This system of particle removal depends on the settling of debris on the mucus carpet in the respiratory airways. The cilia that move the mucus crainiad arise from the epithelium which secretes mucus. This protective layer of mucus can vary in composition and consistency with respect to the ratio of mucopolysaccharides from highly viscous to serous, thereby affecting the mobility of the mucus (Breeze 1985) . Hydration of the mucociliary escalator is of great importance in maintaining a functional and efficient barrier to bacteria and debris. Dehydration, hypoxia, hyperoxia or exposure to caustic irritants (e.g. ammonia or sulfur dioxide) may cause sufficient disruption of cilia and epithelial physiology to inhibit the effective protection offered by the mucocilliary escalator. This will therefore predispose an individual animal to an increased risk of pulmonary infections and diseases (Breeze 1985; Liggitt 1985). Moving through successive bronchiole generations of airways, the number of cilia decline in density (Breeze 1985). This results in a decreased ability to clear mucus from the terminal bronchioles (Wiksie 1985). Other elements of the lung defense system including neutrophils, alveolar macrophages, IgA and lactoferrin are active in the mucoid layer of the upper airway (Dyer 1982; Liggitt 1985). Though important in pulmonary defense, the mucociliary escalator is but one of a myriad of defenses active in the protection of the lungs (Thompson 1974).

The importance of the mucociliary escalator may lie in its ability to maintain a clear passage in the upper bronchioles so as to ensure airway patency and remove pathogenic organisms.

Another physical defense of the more cranial portions (major bronchi and trachea) of the upper respiratory tract is the cough. The cough is a sudden violent contraction of the diaphragm and the abdominal and respiratory musculature that produces a very quick compression of the thoracic cavity and diaphragm against a closed or semi-closed glottis. This results in a forceful expulsion of air through the tracheo-bronchial tree which carries with it debris and mucus which are then eliminated by swallowing the salivary secretions (Dyer 1982; Liggitt 1985).

Immunological defenses of the upper respiratory tract may be divided into immunological tissue-based defenses and immunological secretory defenses. The immunological tissue-based defenses are commonly known as bronchus associated lymphoid tissue (BALT). BALT consists of two types of tissue, lymphoid nodules and lymphoid aggregates. Lymphoid nodules are primarily found in the submucosa of the tracheal tree (Schummer 1979; Breeze 1985; Sminia 1989). The nodules are well organized and contain T lymphocytes (approximately 20%) and B lymphocytes (approximately 50%) while the remainder of the lymphocytes (30%) are unidentifiable (Breeze 1985; Liggitt 1985). Lymphoid aggregates and infiltrates are less well defined and organized, but are often associated with lymphocytes (Breeze 1985; Liggitt 1985; Sminia 1989). BALT appears to be absent in the neonatal calf but increases with age to a peak at approximately 18 months of age and then again decreases (Anderson 1986). BALT is able to initiate local immunological defenses, may elaborate primarily IgG and IgA in cattle and may have other, as yet, unrecognized functions (McDermott 1982; Tizard 2000). BALT communicates through the deep lymphatics that drain the pulmonary tissues and follow the bronchioles as described above (Liggitt 1985).

Secretory defenses of the upper respiratory tract include: IgA, IgG and IgE among other components (Liggitt 1985; Tizard 2000). In a calf, younger than six weeks, there is a predominance of IgG throughout the upper respiratory tract however, after six weeks there is a predominance of IgA in the nasopharyngeal area (Liggitt 1985). IgA, produced by B-lymphocytes, predominates in the nasopharyngeal areas of the respiratory tract and protects the animal by 1) preventing the adherence of particles to the mucosa, and 2) enhancing pinocytosis (Breeze 1985). The predominant secretory antibody of the bronchioles is IgG which protects the respiratory tract by opsonization of the invading organism (Liggitt 1985; Tizard 2000). IgE mediates local allergic reactions and in the event of its activation goes on to enhance the formation of IgG (Tizard 2000). Interferon, another of the secretory defenses, is elaborated in the nasal mucosa (Liggitt 1985). Its primary activity is to inhibit the replication of foreign organisms (including DNA and RNA viruses) by interference with nucleic acid synthesis (Tizard 2000).

Cellular defenses of the upper respiratory tract include: neutrophils, macrophages and lymphocytes which are interspersed among the mucopolysacharides of the mucociliary escalator (Dyer 1982; Liggitt 1985; Tizard 2000). Neutrophils act primarily by phagocytosing and killing bacteria by proteases and free radical generation (Liggitt 1985). Their numbers measure less than 2% of fluid collected by bronchial alveolar lavage, however, vascular neutrophils (up to 50% of circulating leukocytes) may respond to the chemotactic signals released by activated vascular and pulmonary alveolar macrophages and IgG thereby augmenting the numbers of PMN's in the bronchioles (Liggitt 1985; Tizard 2000). Macrophages are also found in the mucociliary escalator fluid though in much lower numbers than is found in the lower respiratory tract (Liggitt 1985; Tizard 2000). They also attract other cellular defenses by the elaboration of cytokines (particularly IL-8), immunoglobulins (primarily IgG), chemo-attractant factors and vasoactive amines to initiate inflammation (Liggitt 1985; Tizard 2000). Lymphocytes may also be active in the upper respiratory tract. These are B lymphocytes, which exist primarily in the BALT and act to generate IgA, and

attract T lymphocytes. Though few in number, lymphocytes serve as an anamnestic surveyor of the antigens present (Liggitt 1985; Tizard 2000).

The lower respiratory tract defenses (terminal bronchials and alveolae) are predominated by the activity of the cellular defenses. The primary cell types include: pulmonary alveolar macrophages (PAM) and lymphocytes (T cells and B cells). The pulmonary alveolar macrophages act to phagocytose and kill microorganisms (Breeze 1985; Liggitt 1985; Tizard 2000), lymphocytes are primarily active in immunosurveillance and defense as described above. The immunoglobulins most commonly noted are IgG and IgA (Liggitt 1985; Tizard 2000).

Pulmonary alveolar macrophages (PAM) lie in a thin layer of surfactant in the alveolus of the lower respiratory tract and play a primary role in chemotaxis, phagocytosis, antigen processing and elaboration of cytokines (Breeze 1985). These cells are derived from monocytes and interstitial macrophages (Bowden 1984; Liggitt 1985). PAMs interact with neutrophils and eosinophils of the lower respiratory tract. The PAMs are approximately 80% of the cells found at this level and is the primary defense mechanism of the alveolus (Trigo 1984; Liggitt 1985). Macrophage defense represents a double edged sword. Elaboration of antibacterial oxygen free radicals and cytokines can have a damaging effect on the pulmonary microvasculature and airway functioning (Engels 1985; Barnard 1992; Seccombe 1994) . Chemotactic factors, chemical radicals and cytokines act both to protect the alveolus and incite inflammatory processes and coagulation within the lumen of the alveolus, the inter-lobular septa and at the immediately adjacent vasculature (Breeze 1985; Yoo 1995). An inflammatory response, at this level, can decrease dynamic compliance of the lung so as to initiate or facilitate in the obstruction of the terminal bronchioles. There is evidence that pulmonary alveolar macrophages can increase in number as a result of increased stress. This could lead to an enhanced defensive and inflammatory response (Boorman 1979; Bowden 1980). Further the macrophage can divide in-vivo to form a self sustaining pool of cells (Boorman 1979; Bowden 1980; McGuire 1982). The optimal PAM : bacteria killing ratio has been reported to be on the

order of 10:1 and effective killing was reduced when the ratio was reversed to 1:10 (Trigo 1984). This suggests that optimal numbers of PAM must be closely regulated and if diminished could result in a significant lapse of microbial defense at the level of the alveolus.

In an anaerobic environment it has been demonstrated that superoxide anion release is decreased while lysozyme release and phagocytosis was unaffected in the killing of *Candida albicans* (Thompson 1992). Under anaerobic conditions macrophages, stimulated in-vitro with phorbol myristic acetate, have been shown to be able to survive for up to 48 hours, if reintroduced to a normal oxygen level within that time. Macrophages in an anaerobic environment show increases in production of lactic acid and glucose uptake, at 24 hours, there is cell degeneration as demonstrated by decreases in the level of cellular ATP (Cazin 1990; Thompson 1992). However, macrophages are unable to recover either homeostasis or protective functioning after 72 hours in an anaerobic environment (Cazin 1990). One of the macrophage antimicrobial functions is to elaborate cytokines (Engels 1985; Kelly 1990; Benburnou 1992; Ward 1993; Yoo 1995). The elaboration of cytokines, primarily Tumor Necrosis Factor-alpha [TNF-alpha] and Interleukin-1 B [IL-1B], is arrested in anaerobic conditions (Matthews 1987). This information again suggests that in an anaerobic environment primary alveolar defenses are reduced or abrogated dependent on the length of time the anaerobic conditions are present.

The functions of TNF alpha are numerous and complex in an in-vivo system. TNF-alpha is specifically formed in response to endotoxin challenge (Sharma 1992) and is also elaborated by vascular endothelium, smooth muscle and other cellular elements (Warner 1989). TNF-alpha acts to stimulate the production of IL-1 and class 1 MHC antigens (Hamblin 1993). By its presence, TNF alpha can generate oxygen free radicals though this can be inhibited by anaerobic conditions (Matthews 1987). It has been demonstrated that TNF-alpha, acting through oxygen free radicals (Matthews 1987; Larrick 1990; Hamblin 1993) can cause significant vascular, endothelial and interstitial tissue injury (Barnard 1992; Seccombe 1994). TNF-alpha may also modify

vasoreactivity through the inhibition of nitric oxide, a potent member of the endothelial relaxing factor family (Johnson 1992). Il-1 stimulates B and T lymphocyte activity, activates neutrophils, vascular endothelial cells and its release is also stimulated by endotoxin and inhibited by drugs with antioxidant properties (Ku 1990).

The neutrophil, although initially in low numbers in the alveolus, actively responds to the chemotactic signals of: IL-8, complement and some arachidonic acid products (Liggitt 1985; Hamblin 1993). The neutrophil has a beneficial effect in the reduction of direct tissue damage by PH through its antibacterial activity (Breider 1991). The presence of the neutrophil also has the potential to contribute to both alveolar and vascular tissue damage through the generation and release of free radicals (Vandenbroucke-Grauls 1987; Warren 1987; Jaeschke 1990; Maheswaran 1993; Granger 1994). The significance of neutrophil mediated damage has been assessed and can be shown to be reduced in neutrophil deficient animals (Breider 1988; Paller 1989).

Other cellular elements of the lower respiratory tract include T lymphocytes and B lymphocytes that consist approximately of 10% of the total free cells found in the normal bovine lung (Liggitt 1985). The function of T cells includes: the activation of PAMs to act as T helper and T suppresser cells as well as T cytotoxic cells (Breeze 1985; Liggitt 1985). PAMs with T cells, act as antigen presenting cells, to ensure an appropriate anamnestic response (Liggitt 1985; Tizard 2000). The B cell generates IgA which is of minor importance in the lower respiratory tract as compared to the nasal pharyngeal (Liggitt 1985). Some plasma cells elaborate IgG. This is the predominant antibody of the lower respiratory tract of cattle (Liggitt 1985). IgG serves to opsonize antigens and enhance bacterial phagocytosis (Liggitt 1985; Tizard 2000). The secretory products of the lower tract are intimately tied to the cellular defenses already described.

Complement is a protective element that can, in early inflammation, be found throughout the lung including the alveoli (Liggitt 1985). The presence of the complement components C5A and

C3A (the anaphylactoids) have been shown be an effective antibacterial mechanism even at low concentrations. These anaphylactoids may initiate tissue damage through the mediation of inflammation and coagulation (Liggitt 1985; Parke 1995; Tizard 2000). Other secretory products of the lower respiratory tract include alpha-1-antitrypsin, alpha-2-macroglobulin, fibronectin, lactoferrin and surfactant (Liggitt 1985).

E) Pulmonary Vascular Control Mechanisms

The autonomic nervous system is primarily involved in the control of the pulmonary vasculature. Three systems are interactive in vascular control and include the cholinergic, adrenergic and non-adrenergic-non-cholinergic (NANC) systems (Barnes 1995). These systems are not only involved in the regulation of homeostasis, but also have significant input into airway and vascular dynamic control. Since the primary focus of this dissertation is to investigate the vascular dynamics of the beta adrenergic receptor, only cursory descriptions will be given of the non-adrenergic and extra-vascular functions of these autonomic systems.

Cholinergic innervation of the vasculature has been reviewed in the bovine and other mammals (Fisher 1965). The physiological significance of this system on vascular physiology is questionable (Bergofsky 1979; Lefkowitz 1991). Paradoxical, simultaneous dilation and contraction of pulmonary vessels to acetylcholine suggests the complexity of this mechanism of control. These findings suggests a "vernier type" vascular control mediated by the action of the cholinergic system on the pulmonary vasculature (Bergofsky 1979).

Nonadrenergic noncholinergic (NANC) mechanisms are also active in the neural-humoral control of the pulmonary vascular control (Barnes 1986). This control is mediated by the simple molecules and neurotransmitters, nitric oxide (NO) in cattle and rabbits (Gustafsson 1991) and vasoactive intestinal peptide (VIP) in cattle (Barnes 1986). These two agents act to cause

vasodilatation in response to their release. Substance P, also a neurotransmitter of the NANC system, has been shown to cause vasodilatation in pigs (Adnot 1989).

Adrenergic control is the third neurological based pulmonary vascular control mechanism and the focus of this dissertation. The adrenergic system is divided into three types of activity based on the receptors mediated and its pharmacological specificity. First, the alpha-1 adrenergic receptor system, defined pharmacologically as responding to alpha-1 adrenergic agonists (e.g. phenylephrine, methoxamine) and blocked by alpha-1 adrenergic antagonists (e.g. prazosin). The typical vascular alpha-1 adrenergic receptor acts to cause constriction of the pulmonary vasculature (Ruffolo 1991). The alpha-2 adrenergic receptor system responds to alpha-2 adrenergic agonists (e.g. clonidine) and is blocked by alpha-2 adrenergic antagonists (e.g. yohimbine, rauwolscine) (Ruffolo 1991). The vascular function of the alpha-2 receptor is not completely clear and appears to be more complex than that of the alpha-1 receptor. Alpha-2 receptors are found directly on the endothelium, on vascular smooth muscle and pre-synaptically as well (Lefkowitz 1988). The alpha-2 receptor appears to have a dualistic nature in its activity. It may act to cause vascular relaxation in the presence of high alpha-1 pulmonary vascular tone or can result in vascular contraction in the presence of low alpha-1 vascular tone. The beta receptor responds to beta adrenergic agonists (e.g. isoproterenol or terbutaline) and may be blocked by beta adrenergic antagonists (e.g. propranolol). The action of the beta receptor is to induce vasorelaxation in the pulmonary vascular bed (Lefkowitz 1974). The beta adrenergic receptor, its functioning or dysfunction with respect to pathogenesis of pneumonic pasteurellosis will be the primary focus of this dissertation.

All adrenergic receptors are G-protein based receptors. The alpha-2 and beta receptors act through a common mechanism of adenylyl cyclase, cAMP / cGMP modulation. The alpha-1 receptor acts through the inositol triphosphate / diacylglycerol (IP_3 / DAG) mechanism (Ruffolo 1991). The specific effect of each adrenergic receptor type is variable with respect to its anatomical location.

Alpha-1 adrenergic system may be activated by selective agonists (phenylephrine, methoxamine) (Ross 1996) resulting in pulmonary vasoconstriction (Limbird 1985; Pepperl 1994). The molecular mechanisms of this stimulation are mediated by G-protein coupled phospholipase C (PLC). This generates two biochemical products, diacylglycerol (DAG) and inositolphosphate (IP_3). DAG phosphorylates a protein kinase and IP3 acts directly to increase intracellular calcium to initiate smooth muscle contraction . It has been demonstrated that alpha-1 receptors are dependent on both intracellular calcium (Ca^{++}) stores and extracellular Ca^{++} concentration to induce maximal vasoconstriction (Ruffolo 1991; Hoffman 1995; Hoffman 1996; Ross 1996).

Alpha-2 adrenergic receptors may be activated by an alpha-2 specific agonist (e.g.. clonidine). Clonidine is considered selective for the activation of the alpha-2 receptors at physiological or low dose pharmacological levels, though it does have some weak alpha-1 agonistic activity (Ross 1996). Alpha-2 receptors are found on both the pulmonary endothelium and the vascular smooth muscle (VanHoutte 1989).

The presence and actions of the alpha-2 receptors in the aortic tissue of rats has been brought into question (Randriantsoa 1981; Dashwood 1985; Martinotti 1991). Dashwood and Jacobson stated that there were no alpha-2 receptors in the aortic tissue of Wistar rats by autoradiographic ligand binding studies (Dashwood 1985). Some questions have been raised about the use of autoradiographic ligand binding studies for alpha-2 adrenoreceptors in view of their rarity on the aorta of the rat (Randriantsoa 1981). The results of Dashwood and Jacobson may be affected by factors that have been known to effect the expression of other receptors including age, species, breed, strain and sex. Martinotti suggests that there are no alpha-2 receptors in the aortic tissue of 200-220 gram, male, Sprague-Dawley rats though their method of receptor evaluation is less rigorous than that of the previous study by Dashwood and Jacobson. Martinotti concluded, based on the lack of response to detomidine (an alpha-2 receptor agonist), that there are few or no

alpha-2 receptors in aortic tissue of the rat (Martinotti 1991). However Martinotti's experiment could represent “zero sum” pharmacological response to an alpha-2 agonist and partial alpha-1 agonist described above. The results of Randriantsoa suggests that the alpha-2 receptor exists in the rat aorta but may represent a variation in response from a true alpha-2 receptor (Randriantsoa 1981).

In light of these findings, it is likely that the alpha-2 receptor (or possibly a variation of the alpha-2 receptor) are rare in the rat aorta, though their importance cannot be measured by their numbers but is apparent by their physiological functioning in-vivo. It is possible that this receptor acts to buffer the extreme constrictions mediated by the alpha-1 receptor. Further, the alpha-2 receptor may, in fact, be the vascular equivalent of the "fine tuning" knob balancing the effects of beta-mediated vasodilation and the alpha-1 mediated vasoconstriction. These possibilities have yet to be investigated experimentally. Another possible explanation for the dual nature of the alpha-2 receptor is that the class of receptors responding to an alpha-2 agonist are variable or heterogeneous in their presence, numbers and activity on the endothelium of the rat aorta and vary only in homeostatic importance only in response to some as yet unrecognized signal. It remains to be discovered what, if any, factor affects the presence and functional expression of the alpha-2 receptor in the rat aorta.

The beta receptor agonist, isoproterenol, induces significant vasorelaxation in the presence of high vascular tone in both rat aorta and bovine pulmonary vasculature. High vascular tone may be induced by preconditioning vessels with potassium (e.g. KCL) a cell and membrane depolarizing agent. The mechanism by which beta receptor mediated relaxation occurs, in high vascular tone, is through the activation of adenylylate cyclase via a subunit of the G-protein of the receptor. There is a subsequent generation of the second messenger cyclic adenosine monophosphate (cAMP). The cAMP is then thought to activate a protein kinase (myosin light chain kinase) resulting in

dephosphorylation, Ca^{++} sequestration and subsequent vasorelaxation (Hoffman 1995; Hoffman 1996). The beta receptor is discussed in greater detail in the next section.

F) Beta Receptor Mechanisms

G-protein receptors represent a superfamily of receptor proteins that are associated with a signal transducing guanosine molecule. Each receptor (alpha-1, alpha-2 and beta) is an integral membrane protein characterized by seven transmembrane alpha-helical segments (Watson 1994). All adrenergic receptors (alpha and beta) are of the same basic structure with some differences in their amino acid composition and secondary messenger system (Lefkowitz 1991; Hoffman 1995). There is similarity among the groups of adrenergic receptors. For instance, alpha-1 and alpha-2 receptors have approximately 45% homology in their amino acid sequence (Langer 1989). It is likely that there is less homology between beta receptors and their alpha counterparts.

The beta adrenergic receptor polypeptide string consists of seven transmembrane alpha-helices that are joined by short chains of amino acids (Cuatrecasas 1974; Lefkowitz 1974). The amino terminus starts outside the plasma membrane and the first a helix spans the cell membrane while the amino acid loop between the first and second a helices enters the cytosol. The second alpha helix is in an anti-parallel orientation to the first a helix. This architecture continues with all alpha helices in an anti-parallel orientation to its immediate adjacent neighbors. This leads to an accordion type structure of the molecular biology of the G-protein receptor (see figure #2) (Lefkowitz 1988; Levitzki 1988; Ostrowski 1992; Strader 1994).

The loop between the alpha helix #5 and #6 is elongated and plays an essential role in the activation of the adrenergic intracellular mechanisms. Specific amino acids on the #5-6 loop are available to be phosphorylated (the catalytic site) by way of the interaction of an adrenergic agonist with the receptor protein (Lefkowitz 1988; Levitzki 1988; Ostrowski 1992; Strader 1994).

Activation of the beta receptor occurs when an agonist interacts at three separate locations simultaneously, with receptor amino acids of the transmembrane alpha helices. The amino group of the agonist forms an ionic bond with the carboxylate side chain of aspartate (A^{113}) in the third a helical segment. This has been verified by demonstrating that a mutation in A^{113} inhibits receptor-agonist interactions (Levitzki 1988). The two hydroxyl groups of the catechol ring of the agonist, forms hydrogen bonds with two serine residues (S^{204} and S^{207}) on the fifth transmembrane alpha helical segment. Mutation of either of these two serines can also result in the inhibition of the receptor-agonist binding. Finally, the third interaction is between the agonist and the sixth transmembrane helix. The interaction at the catechol ring is the formation of a hydrophobic bond with phenylalanine (F^{290}) of the sixth alpha helix (see diagram of beta adrenergic agonist and antagonist, figure #3), (Lefkowitz 1988; Levitzki 1988; Ostrowski 1992; Strader 1994).

The binding of the agonist to the beta receptor moiety causes movement among the transmembrane helices (particularly #5 and #6). This movement results in the activation of the transducing G-proteins ($G_{a\ stimulatory}$) and subsequent induction of adenylyl cyclase by the catalytic site on the #5 / #6 cytoplasmic loop and finally, in the elevation of cAMP.

The protein receptor moiety is adjacent to but separate from the actual G-protein transducing unit. The G-protein transducing unit consists of the G_g, G_b, and G_a subunits (trimeric unit). These proteins together are referred to as the G_s protein transduction complex. In an inactive form, the G_g, G_b remains bound to G_a and guanosine diphosphate (GDP) is attached to the G_a unit. Once activated, the catalytic site on the cytosolic loop, binds to the trimeric G-protein complex to initiate the intracellular phase of activation. Upon activation, guanosine diphosphate (GDP) bound by the G_a subunit is released and replaced by guanosine triphosphate (GTP) which activates G_a. G_a is an intracellular switch protein that is part of a GTPase superfamily. The activated G_a unit dissociates from the G_g, G_b subunits and binds to adenylyl cyclase at its catalytic domains. Hydrolysis of GTP to GDP allows the dissociation of the Ga from adenylyl cyclase and initiates

the hydrolysis of ATP to cAMP. The now inactive G_a molecule then returns to associate with the G_g, G_b units of the transduction protein.

Adenylyl cyclase is an enzyme bound to the intracellular membrane adjacent to the receptor. Activation of adenylyl cyclase by an agonist results in the intracellular increase of cAMP. An antagonist, on the other hand, either fails to cause a conformational receptor change or initiates a conformational change in the receptor or G-protein moiety that results in a non-functional , non-activated G-protein complex. The most current thinking, with respect to antagonists, is that GDP fails to be replaced by GTP in the G_a molecule, this results in a failure to activate adenylyl cyclase. The gram negative bacterial product, pertussus toxin, acts to lock G_a-GDP complex in a bound state which directly causes receptor functional uncoupling (Levitzki 1988; Simon 1991; Birnbaumer 1992; Hepler 1992; Lodish 1995).

The activation of adenylyl cyclase is one step in the cascade from the receptor-ligand interaction to the final cellular effect (Lefkowitz 1988; Levitzki 1988). It is important since it is a step where there may be amplification of the receptor signal (Levitzki 1988; Tang 1992). In the

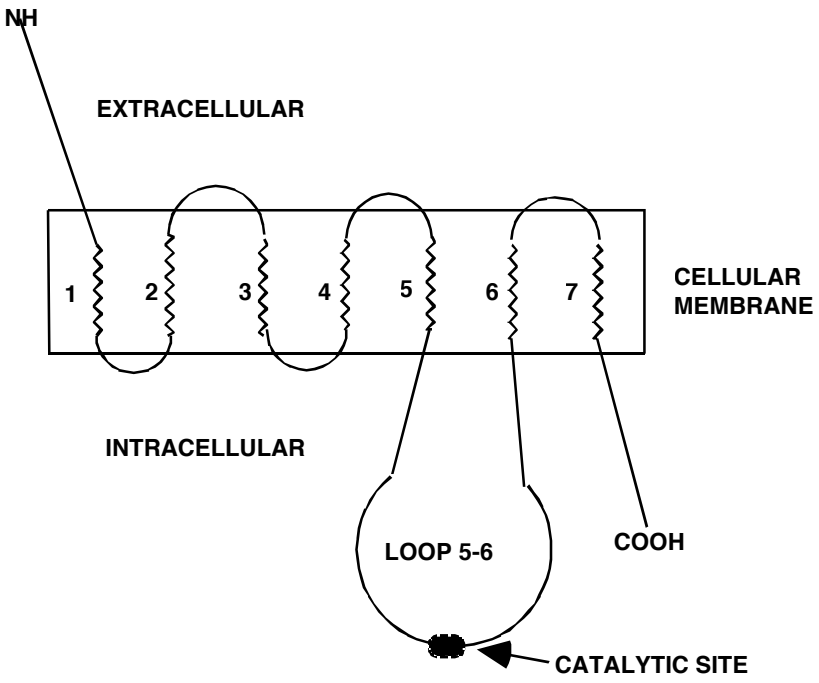


FIGURE #2 - G-Protein receptor with seven anti-parallel alpha-helices. This is the basic format for adrenergic receptors of the pulmonary vasculature.

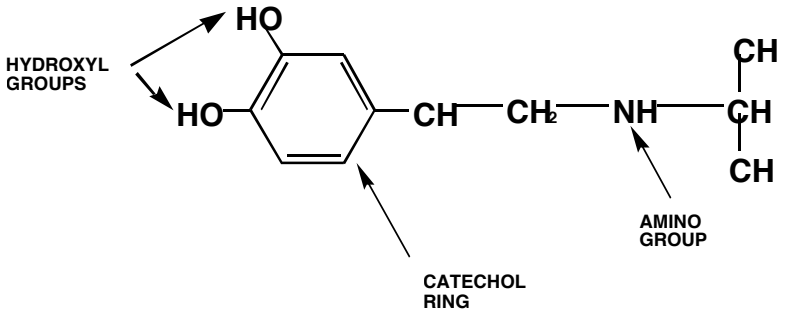
case of the beta receptor, cAMP has a direct effect on the cAMP-dependent protein kinases in the cytosol. These protein kinases, once activated, act to modify cellular activity. Each protein kinase has two regulatory units and two catalytic subunits. The binding of cAMP to each of the regulatory subunits results in the activation of the catalytic subunits which then result in the phosphorylation of specific substrate proteins.

In vascular smooth muscle the phosphorylated proteins modulate intracellular calcium, resulting in relaxation. Calcium is an essential ingredient in both the contraction and relaxation of vascular smooth muscle (Morgan 1991). Calcium enters the cytoplasm from either the extracellular space or from sequestered intracellular stores. In a smooth muscle contraction, the presence of calcium allows the activation of the myosin light chain kinase (MLCK). The MLCK then acts to phosphorylate the myosin light chain which then promotes the myosin-actin cross bridging in the presence of the activated myosin-ATPase. Relaxation occurs by the deactivation of the myosin

through dephosphorylation of MLCK and by the sequestering of Ca^{++} secondary to the action of cAMP on the cGMP-dependent protein kinase (Lincoln 1991). Dephosphorylation is governed by factors that are not tied solely to the intracellular calcium level. Further, it has been found that beta adrenergic agents not only modulate relaxation but also may increase the level of Ca^{2+} necessary for MLCK activation (Morgan 1984).

Among the many types of receptors located on a neutrophil, the beta-adrenergic receptor is functionally most important. It is reported that there are up to 10^6 stimulatory receptors, including those for complement degradation products, formyl peptide and immune complexes, on the PMN cell surface. Further, there are approximately $2x10^4$ inhibitory receptors, including receptors for prostaglandin E_1, and beta adrenergic agonists. Beta receptors are by far the minority: approximately 10^3 (5%), of the inhibitory cell surface receptors and .04% of the entire receptor population on the neutrophil (Galant 1981; Galant 1984; Sklar 1986; Mueller 1988). The beta-2 receptor is the primary adrenergic receptor found on the leukocyte (Motulsky 1982). Beta receptors have been proposed to be one mechanism for neutrophil and macrophage regulation of the release of oxygen free radicals (Engels 1985; Ogunbiyi 1986; Borregaard 1988; Barnard 1992; Opdahl 1993; Seccombe 1994), phagocytosis, chemotaxis, and granular enzyme release (protease, and beta-glucosidase) (Bourne 1971; Ignarro 1974; Zurier 1974; Hills 1975; Busse 1984; Tecoma 1986; Al-Essa 1995). More recently it has been demonstrated that, by activation of beta adrenergic receptors on both neutrophils and the eosinophils, it is possible to inhibit leukocyte adhesion to venules in the rat trachea and thus reduces the amount of plasma leakage to perivascular tissues (Bowden 1994). The beta adrenergic mediated inhibition of exuberant inflammation by circulating cells is thought to be important in limiting surrounding tissue destruction (Mueller 1988). Confirmation that beta receptor mediated leukocyte inhibition occurs was demonstrated by the restraining effects beta adrenergic agonists had in-vitro and that this activity could be blocked by propranolol, a beta adrenergic antagonist (Tecoma 1986; Mueller 1988; Opdahl 1993). Further, it was established that when leukocyte receptor affinity for classical adrenergic agonists was

CLASSICAL AGONIST- ISOPROTERENOL



CLASSICAL ANTAGONIST- PROPRANOLOL

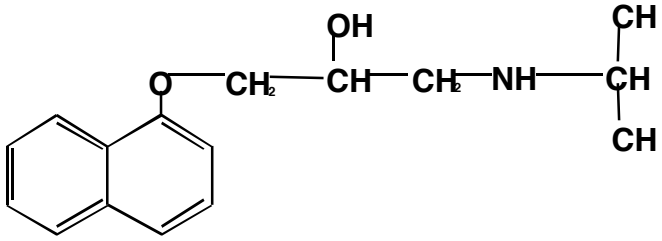


FIGURE #3- Structures of a classical agonist, isoproterenol and non-depolarising antagonist propanolol. Note that the antagonist lacks the hydroxyl groups on the catachol ring.

examined, the relative affinities were identical to those of other classical beta receptors; isoproterenol > epinephrine > norepinephrine (Galant 1981).

The ED_{50} for isoproterenol, a beta adrenergic agonist that induced changes in human neutrophil beta receptors, has been calculated to be approximately 3 x 10^{-8} M (Mueller 1988) to 1 x 10^{-7} M (Tecoma 1986). These values are calculated for an in-vitro preparation, however it is likely that endogenous catecholamines act at the lower "physiological" level at the receptor site in-

vivo. There is a difference in the ability of beta agonists to inhibit neutrophil free radical generation in circulating blood-borne neutrophils when compared to their tissue based counterparts (Al-Essa 1995). In circulating neutrophils, at least 30-40% of the beta receptors on this leukocyte must be occupied to have an inhibitory effect on the generation of oxygen free radicals (Mueller 1988; Opdahl 1993; Al-Essa 1995). This is amazing since 40% occupancy of neutrophil beta adrenergic receptors represents approximately .04 % of the total neutrophil receptor population (calclated estimate). This small number of specialized receptors hold the ability to mediate the release of oxygen free radicals over-ruling over 99% of the remaining neutrophil receptors! Further, this suggests two significant points: first, a minimal disruption of beta adrenergic receptor complex could have significant physiological and pathological effects and two, naive neutrophils need minimal endogenous beta adrenergic agonist activity to control the release of oxidative free radicals. Finally, pharmacological levels of activators are likely to overstate the more subtle endogenous activity of normal physiological levels of beta adrenergic mediators in the vasculature.

Minimal dysfunction of the neutrophil receptor population may lead to unchecked generation of oxygen free radicals. The definition of minimal dysfunction in this case is, as yet, to be experimentally assessed and defined. The disruption of beta receptor mechanisms following prophylactic vaccination, using live *Pasteurella haemolytica*, of the vascular smooth muscle has been described by Weekley (1993E). A damaged vascular beta receptor likely results in a diminished functional population of receptors in the presence of an increased vascular tunica muscularis tone (as is the case in the bovine pulmonary vasculature). This can result in excess venous constriction due to the unchecked action of intact alpha-1 receptors and alpha-2 induced contraction (Weekley 1993E). Similarly, beta receptor uncoupling in the leukocyte population, particularly the neutrophils, has the potential to result in an increased release of PMN generated oxygen free radicals and consequently severe pulmonary tissue derangement, damage and destruction.

Beta receptors are also found on the vascular endothelium and have been demonstrated to modify extra vascular fluid movement (Mizus 1985; Minnear 1986) and inhibit the coagulation system through the modification of tissue factor (Busso 1991). Disruption of these receptors, in the pulmonary vasculature, could result in pulmonary vascular leakage resulting in tissue congestion, and activation of the coagulation cascade resulting in deleterious thromboembolic events.

Given the significant and intimate involvement of the beta adrenergic receptor in the pulmonary vascular homeostatic mechanism, it would appear that the uncoupling of these receptors could be an essential element in the development of pulmonary pathophysiology in bovine pneumonic pasteurellosis.

G) Significance of Beta Adrenergic Receptor Dysfunction in the Pathogenesis of Bovine Respiratory Disease Complex and Pneumonic Pasteurellosis

Pneumonic pasteurellosis is characterized by two distinct sequential pathological events, the first related to the "invasion of the respiratory system" either singly by the organism *Pasteurella haemolytica* as an opportunistic infection, or in concert with a secondary bacterial or viral vector as has been described previously. The second or perhaps the primary insult is that of tissue inflammation in the respiratory tract. The hypothesis of this dissertation implies the importance of the beta adrenergic receptor in the mediation and regulation of the inflammatory aspect of the disease process of BRDC or pneumonic pasteurellosis. By extension the suggestion is that the loss of regulation of the pulmonary inflammatory process, as a consequence of the disruption of the beta adrenergic receptor can result in the excessive damage to bovine lungs as is commonly noted in this disease process. Inflammation is a complex event that is a consequence of tissue derived mediators that, when uncontrolled, can account for all of the signs and indications of the morbidity and mortality of pneumonic pasteurellosis. Scientific acceptance of this thesis may give more

specific direction to the development of a new prevention and treatment strategies that ultimately could result in great improvements in the cost, both pathologically (to the individual animal) and financially (to the producer), of this devastating disease. I would like to first review the specifics of the inflammatory process, then suggest the role of the beta adrenergic receptor to this process. This understanding may then suggest possible future targets of treatment that could prove invaluable in the prophylaxis, metaphylaxis and treatment of pneumonic pasteurellosis in cattle.

The inflammatory process involves multiple physiological systems including: leukocytes (primarily neutrophils), pulmonary alveolar macrophages, platelets, T-cells, mast cells, cytokines among other factors. Also intimately involved in the pulmonary inflammatory response are several cascade, systems including but not limited to, the arachidonic acid cascade, the coagulation cascade, the complement cascade and finally the kinin cascade. These two lists of endogenous systems are not meant to be a comprehensive compilation of all endogenous systems involved in pulmonary inflammation, but merely a brief overview of what are likely to be the more important systems involved with pneumonic pasteurellosis. The majority of these systems are integrated into a complex of inter-related physiological processes at the center of which is the overall modulating transcription factor, Nuclear Factor kappa B (NF kappa B). The basic elements of pneumonic pasteurellosis are infection, endogenous inflammatory mediators, Nuclear Factor kappa B and the beta adrenergic receptor.

Many of the lesions described both on gross and microscopic pathological examination can be explained in terms of pulmonary vascular homeostatic dysfunction in general, and beta adrenergic uncoupling in particular. Pneumonic pasteurellosis can lead to either fibrinous bronchopneumonia or fibronecrotic pneumonia with a lobar pattern. Typical initial gross pathological lesions include: reddish-black to dark brown-gray consolidated cranial ventral lung lobes, thickening of the interlobular septa, fibrinous pleuritis, the thorax may contain a straw colored effusion and areas of necrosis can be seen in the lungs (Rehmtulla 1981; Dungworth 1983; Wiksie

1985; Lopez 1986). In most cases resulting in mortality, the gross lesions involve 60-80% of the total lung (Wiksie 1985). Histopathological lesions include: necrosis with large numbers of bacteria, thrombosis and leukocyte accumulation have also been noted (Rehmtulla 1981; Dungworth 1983; Wiksie 1985; Lopez 1986)**.** The pathology described above maybe associated with events initiated by the disruption of the beta adrenergic receptors of the pulmonary vascular smooth muscle, endothelium, and circulating leukocytes. This may result resulting in uncontrolled exuberant inflammatory activity.

Inhibition of the functioning of the mucociliary escalator is likely one contributing factor to the pathology seen in BRDC (Breeze 1985). Factors such as metabolic or respiratory acidosis can lead to decrease in the efficacy of the mucociliary escalator (Breeze 1985; Liggitt 1985) . The mucocilliary protective system may also be interrupted by viral infections in both the bovine (Breeze 1985; Hjerpe 1986; Radostits 1994) and in the equine (Dixon 1992). The interruption of this physical defense could result in a gravitational dependent drainage to the anterior ventral aspects of the lung. This is likely to form multiple mucus plugs in the terminal bronchioles which would then isolate the associated acinus. Without collateral communication (via pores of Kohn) in the bovine lung, an anaerobic environment is established. This would likely change the nature of any PH trapped in this environment (Leeper et al 1992, Christman 2001).

In an in-vitro anaerobic environment, PH demonstrates an enhanced toxicity (Waurzyniak 1994, Chang 1987). The proposed increase in PH toxicity is thought to be due to an increase in the local expression of leukotoxin seen in the early log growth phase (in-vitro) (Waurzyniak 1994, Chang 1987). This activity declines in the late log to stationary phase of in-vitro bacterial growth (Chang 1987). These findings support the labile pathogenic nature of PH. To date, only two studies have been completed on the characterization of PH endotoxin in an anaerobic environment. The first study (Davies 1992) examined the quantitative changes under anaerobic conditions in-vitro and found a decrease in the quantity of LPS. This study is lacking on two essential points, first the

dynamic and complex interactions of multiple factors including: cytokines, immunological chemokines, vasoactive autocoids and tissue factors, that in the anaerobic, acidic acinus are unlikely to be duplicated in-vitro. Secondly, an in-vivo environment is likely to be a facultatively anaerobic environment rather than a true anaerobic situation. In another investigation, it was shown that bovine serum albumin (BSA) is a factor in the elaboration of LPS (Davies 1992). Bovine serum albumin may be elaborated as a consequence of BRDC when PM infects the alveolus (Waurzyniak 1994). As noted previously, the qualitative nature of rough and smooth endotoxin in PH and the different effects these two types of endotoxin have on cyclic nucleotides is great (Emau 1987), however no evaluation has been made as to the relative amounts of rough and smooth endotoxin under anaerobic or aerobic conditions. In a second study, Wessman showed that there is variation in rough and smooth cell variants in varying oxygen tensions. The more virulent smooth cell colonies were dominated by the less virulent rough colonies in low oxygen tension (Wessman 1964). In combination, the two studies cited above, suggest that the elaboration of a more virulent LPS is possibly under anaerobic conditions. This is an indication that changes in the virulence of PH with respect to the alveolar micro-environment, remains to be investigated in-vivo. It is likely that the severity of the beta adrenergic receptor dysfunction is related to qualitative changes in PH.

Leukocytes are important in the pathology associated with pulmonary inflammation in general and pneumonic pasteurellosis specifically and are acted upon by, PAF, IL-8, LPS and many other endogenous and exogenous mediators. It has been suggested that microvascular damage to endothelial cells by neutrophil generated elastase is enhanced by endotoxin (Smedly 1986). It has also been shown that endotoxin can enhance pulmonary vascular sequestration of leukocytes in sheep (Sigurdsson 1989).

The LPS mediated adrenergic receptor disruption has an effect on circulating and tissue leukocytes (Slocombe 1985; Breider 1988). The oxygen free radical release by neutrophils is diminished in the presence of a beta receptor agonist (e.g. isoproterenol) (Engels 1985; Ogunbiyi

1986; Borregaard 1988; Barnard 1992; Opdahl 1993; Seccombe 1994). This results in an unmodulated increase in the generation of oxygen free radicals. Events of this nature have been shown to enhance tissue damage in the micro-environment in other organs, species and in-vitro (Bourne 1971; Ignarro 1974; Zurier 1974; Hills 1975; Harlan 1981; Busse 1984; Engels 1985; Tecoma 1986; Warren 1987; Barnard 1992; Kubes 1993; Granger 1994; Seccombe 1994; Al-Essa 1995). Evidence for the prominent role of neutrophils in the histopathology of respiratory disease has been noted. Neutrophil deficient animals suffer less vascular and pulmonary damage than neutrophil sufficient animals with respect to the lungs in calves (Slocombe 1985; Breider 1991; Maheswaran 1993), in the kidneys (Paller 1989) and liver (Jaeschke 1990) of rats. However, the authors investigating neutrophil mediated pathology failed to examine the effects of beta adrenergic agonists and antagonists in modulating the neutrophil.

Other cellular elements such as thymic derived lymphocytes or T-cells have been recognized as being involved in pulmonary airway allergic inflammation (Stirling and Chung 2000). Though pneumonic pasteurellosis is unlikely to be mediated by airway allergic mechanisms this may be one of the many pathways induced that leads to pulmonary and vascular inflammation.

There are three types of T-lymphocytes: the Th-0 (naive cells), as well as the two activated cells Th-1 and Th-2 (Tizard 2000). The naive T-cells are induced to activity by the presentation of an antigen by an antigen presenting cell (APC). This then results in the activation of two equally important co-stimulatory receptors on the Th-0 cell, the CD 4^+ and the CD 28 receptor (Green 1982). The antigen presenting cell that is most likely involved in this process is the pulmonary alveolar macrophage. The activated Th-0 cell is then transformed into either a Th-1 cell, in an IL-12 and interferon-gamma predominated environment or a Th-2 cell in an IL-4 environment. The Th-1 cells are a natural counterbalance to the Th-2 pro-inflammatory cells (Stirling 2000). Successful generation of the Th-2 cell requires that the APC activates both the CD 4^+ and the CD 28 receptor of the naive T-cell (Harding 1992). The distinction between the Th-1 cells and the Th-2 cells is

related to the cytokine they differentially secret (Mosmann 1986; Mosmann 1991). Failure to activate both T-cell receptors (TCR) would result in a less than efficient Th-2 immunological response (Humbert 1999). The Th-2 cells generate multiple cytokines (including IL-4, IL-5, IL-9, IL-10, and IL-13) that promote inflammation and the further generation of the Th-2 cell type of lymphocyte. The IL-4 cytokine promotes the generation of the Th-2 lymphocyte preferentially over the Th-1 lymphocyte. Airway inflammation is mediated through mast cell activation, immigration of eosinophils and IgE (Chung 1999) as well as the activation of surface markers and the increase in goblet cells resulting in increased intra-luminal mucus (Kips 1999; Bosquet 2000) all induced by the activities of the cytokines. It is therefore clear that a shift in the balance from the Th-1 to the Th-2 lymphocytes could result in an escalation of pulmonary inflammation. As well, the increased generation of mucus in the bovine lung, could result in isolation of individual alveoli thereby creating an anaerobic environment which would promote changes in pulmonary alveolar macrophages leading to further alveolar and vascular inflammation. Though a factor in the development of pneumonic pasteurellosis, the significance of the various T-cell populations and their activities has yet to be investigated with respect to BRDC.

Control of the Th-1/Th-2 by beta adrenergic receptors has been investigated in murine Thymic helper cells and reveals that there is a differential expression of the beta receptor on the Th-1 / Th-2 cells (Ramer-Quinn 1997). Murine Th-1 resting and activated cells appear to have beta adrenergic receptors associated with the down regulation of the IL-2 cytokine, whereas this does not appear to be the case with respect to the to the Th-2 resting cell (Sanders 1997). IL-2 has been demonstrated to be the initiator of the "vascular leak" syndrome from the lung vasculature (Parke 1995). The results presented for beta adrenergic receptors in murine Th-1 cells are related to the cells found in the lymph nodes and have yet to be confirmed for bovine pulmonary resident Th-1 and Th-2 cells.

Platelet Activating Factor (PAF) has been recognized for its role in pulmonary inflammation (Pinkard 1982) and experimental pneumonic pasteurellosis in calves (McClenahan 2000). PAF has multiple effects including induction of platelet activation, neutrophil aggregation, bronchoconstriction, increasing pulmonary vascular resistance and the initiation of pulmonary edema (Vargaftig 1981; Pinkard 1982; Suquet 1983; Foster 1992; Maxwell 1993). In addition, PAF also is a co-stimulator of TNF-alpha induced PMN release of oxidative factors (von Asmuth 1995) and has a direct effect on pulmonary artery endothelial cells (Grigorian 1987). The implications of the pathophysiology of PAF with respect to pneumonic pasteurellosis in calves has recently been discussed in McClenahan (McClenahan 2000).

The interaction of PAF, IL-8 and activated complement factors are synergistic affect with respect to the intravascular attraction of PMNs. As previously noted, several authors have demonstrated that in neutrophil deficient animals, there are a significantly decreased number of pneumonic lesions in the bovine lungs (Slocombe 1985; Breider 1988). The decrease in lesions parallels the decreases in fibrin, erythrocyte and leukocyte adhesion, septal necrosis and finally decreased albumin concentration in BAL fluid (Slocombe 1985; McAdams 1988; Weiss 1991). PAF also has direct effects on the ex-vivo cells including desensitization of the beta adrenergic receptors on the pulmonary endothelial cells (Grigorian 1987). LPS in concert with PAF are two of the essential elements in pneumonic pasteurellosis. *Pasteurella haemolytica* LPS acts through IL-8 and enhances a PMN margination response in the bovine lung (Yamamoto T.; O. Kayikawa 1998). PAF not only initiates the pulmonary vascular emigration of PMNs but also acts with IL-8 and TNF-alpha to enhance leukocyte emigration and increase the release of PMN oxidative enzymes which leads to pulmonary inflammation (von Asmuth 1995). The emigration of platelets and activated complement fragments initiates the coagulation cascade resulting in the disposition of thrombin and fibrin (von Asmuth 1995; McIntyre 1997). In addition, PAF results in the release of thromboxane, prostacyclin and other cyclo-oxygenase products of the aracidonic acid cycle

(Grigorian 1987; van de Weerdt 1999). In total these endogenous products result in enhanced pulmonary inflammation.

The effects of PAF in the pathophysiology of pneumonic pasteurellosis has been evaluated by the use of the PAF inhibitor WEB 2086 (McClenahan 2000). It has been established that PAF is a major factor in the pathophysiology of pneumonic pasteurellosis. By inhibiting PAF, it has been established that there was a reduction of the lung lesions, attenuation of the increase in micro-vascular permeability and a reduction of PMN infiltration. Some of the effects of WEB 2086 may have an affect on leukocyte deformability, PMN size and CD 11b expression with respect to PMNs (McClenahan 2000).

The complement cascade is activated by both the coagulation cascade and endotoxins prevalent in pneumonic pasteurellosis. The activity of the complement cascade as a mediator of pulmonary inflammation has been extensively investigated (Marks 1989; Foreman 1994; Czermak 1998; Hamacher 1998). Complement is another factor in the overall inflammatory activity of the lung. This cascade is often initiated by LPS and involves the complement components, C5a, C3b, C4b and their respective receptors (Czermak 1998; Hamacher 1998).

It has been demonstrated that complement can mediate LPS induced endothelial and leukocyte adhesion factors (Ward 1989; Varki 1992) and endothelial cell activation (Marks 1989; Foreman 1994). Brauer et al, have elucidated the pro-inflammatory properties of complement induced by endotoxin and mediated by the Membrane Attack Complex (MAC) (Brauer 2000). The components of the complement cascade involved in this pro-inflammatory action is most likely to be the anaphylaxatoxins C3a and C5a (Marks 1989; Foreman 1994) and represent the pro-inflammatory mediators of the complement cascade affecting the lungs.

A third factor C6 is integral to the inflammatory and immuno-pathological events process. Brauer et al identified two groups of rats, one group is the natural condition, with the complement factor C6, and the second group represents animals with a deficiency in this same factor (Brigham 1986; Brauer 1994; Brauer 1995). C6 is the complement factor thought to activate the MAC and this subsequently enhances the production of TNF-alpha and endothelial leakage (Kilgore 1995).

These findings have important consequences with respect to inflammation in lungs. First, the interaction of the complement system in the inflammation of the lung is supported. Second, the technique of an intra-peritoneal injection of PH endotoxin initiates systemic, multifactorial events likely including: induction of cytokines, selectins and induction of neutrophil activity. Finally the conclusions of this study indicate that the pro-inflammatory systems, induced by endotoxin, are very likely redundant. The redundant nature of inflammation is supported by the identification Nuclear Factor kappa-B (NFkB).

Pathological studies have confirmed the presence of both thrombin and fibrin that are results of the coagulation cascade and BRDC. These findings suggest the importance of the regulation / inhibition of the coagulation cascade, but also the importance of the interrelationship and inhibition of the cascade by other elements in sequence of pulmonary inflammation.

The coagulation cascade can be initiated by several elements of the pneumonic pasteurellosis disease syndrome. The most significant element of pasteurellosis that initiates coagulation is LPS, via an increase in the Hageman factor (De la Cardena 1993). The Hageman factor also increases on exposure to denuded endothelium, perivascular cells, smooth muscle, tissue factor via an increase in Factor VIII (Drake 1989), activation by factors of the complement cascade, PMN release of elastase, IL-6. Tissue factor (TF) expression increases in monocytes and endothelial cells due to increases in mRNA, cytokines, IL-1 beta, TNF-alpha (Nawroth 1986; Scarpeti 1989; Fei 1993). The TF gene contains sites of activation by NFkB. These sites also exist

in monocytes and endothelial cells (Mackman 1991; Orthner 1995). The el;aboration of NFkB by reactive oxygen intermediates released by actvated macrophages and neutrophils (Mackman 1991; Orthner 1995), then feeds back to an increase in tissue factor (Muller 1993) enhancing the inflammation in the pulmonary tissue.

The consequence of the coagulation cascade is the generation of thrombin. Thrombin is generated at sites of vascular injury. Thrombin has endogenous effects including: enhanced fibrinolytic activity, anticoagulant activity as well as it stimulates endothelial cell synthesis resulting in an inhibition of platelet activation and an increase in vasodilatation. Further, thrombin is active in promoting inflammation to facilitate the process of wound healing (Carney 1992), increase vascular permeability and increase inflammatory cells at the site of injury (Malik 1986) . The effects of thrombin on vascular associated cells is mediated through the thrombin receptor (Bahou 1993).

The thrombin receptor is a member of the G-protein receptor family and is found on platelets, endothelial cells, smooth muscle cells and fibroblasts (Bahou 1993). The consequences of the activation of the thrombin receptor leads to a dual signaling path resulting in an increased turnover of phospholipase-C mediated phosphoinositol (Huang 1987) and an inhibition of adenylylate cyclase (Huang 1987; Brass 1988; Brass 1991). Thrombin also causes an increase in trans-endothelial permeability which is antagonized by the activation of a beta adrenergic stimulating agent, isoproterenol (Minnear 1993).

Finally nitric oxide is elaborated by endothelial in response to increases coagulation and LPS activity (Minnear 1993, Huang 1987). An increase in nitric oxide mediated vascular relaxation (Tesfamarium 1993) increases selectin expression, and therefore, leukocyte adherence (Shenker 1994; Zimmerman.and Paulson 1994), increases prostacyclin production (Ngaiza 1991) and the availability of Von Willebrand's factor as a result of the increase of the P-selectin (Storck 1995).

Activated beta receptors on the vascular endothelial cells, have also been demonstrated to inhibit the elaboration of tissue factor the first step of the extrinsic pathway of coagulation (Majno 1961; Busso 1991). Investigators have demonstrated that injured endothelial cells release thromboplastin and prothrombin which is converted to thrombin then fibrinogen intravascularly (Majno 1961). The simultaneous occurrence of the disruption of the beta adrenergic receptor function on the endothelial cells and endotoxin induced endothelial damage is very likely to enhance the activation of the extrinsic coagulation system leading to a higher potential for coagulopathy. It has also been demonstrated that in high stress states such as induced by: exercise, crowding, decreased pulmonary compliance or increased ventilation perfusion mismatch, a pulmonary metabolic acidosis may develop which can cause further disruption of neutrophil and vascular beta adrenergic receptors (Davies 1984; Davies 1986; Hendricks 1986; Deitch 1987; Davies 1988). Local metabolic acidosis is a logical consequence of either unbalanced adrenergic mediated vascular constriction, or of intravascular coagulation following exposure to *Pasteurella haemolytica* organisms (Weekley 1991B; Weekley 1993B; Weekley 1999). The possibility of enhanced cytokine expression, particularly IL-8, a neutrophil chemo-attractant (Xing, 1994) may add to the local vascular and interstitial pulmonary damage by recruiting more neutrophils to the local area initiating a degenerating oxidative mediated spiral resulting in severe tissue pathology.

Overall the significance of the coagulation cascade to pneumonic pasteurellosis is that these events can be synergistic with the other pro-inflammatory endogenous events. The coagulation cascade is central to both the infective and inflammatory aspects of pneumonic pasteurellosis. Finally, the significance of the inhibition of adenylylate cyclase by thrombin is equivalent to the inhibition of the beta adrenergic receptor.

Cytokine release and production are initiated by the immediate infection phase of pneumonic pasteurellosis. The initiation of cytokine activity is very likely due to the endotoxin moiety of*Pasteurella haemolytica*. Initially, three cytokines appear to be active in initiating

inflammation. The inflammatory cytokines include IL-1B, TNF-alpha, IL-6. These are released due to the influence of LPS on the pulmonary macrophage (Goldblum 1991). Though the inflammatory cytokines are of importance initially, these cytokines act in concert with a neutrophil attractant cytokine, IL-8. The activity of IL-8 mediates the infiltration of neutrophils to the area of inflammation for phagocytosis. The cytokine cascade doesn't end with these initial events. The inflammatory cytokines can facilitate the release of other cytokines through the activity of LPS, T helper cells, neutrophils and multiple other endogenous elements involved in infection and inflammation. This demonstrates the interactive, interdependent nature of infection and inflammation in pneumonic pasteurellosis in cattle.

It is clear that all of the elements above are intimately related. One event is either initiated by an element of inflammation and infection, or is an immediate or downstream step of a cascade event initiated by inflammation. This thesis is presented to elucidate the importance of the beta adrenergic receptor to the control and or manipulation of the inflammatory consequences of the respiratory disease, pneumonic pasteurellosis. With respect to the overall pathophysiological process of this disease, the inflammatory cascade and consequences is likely of greater importance than infection . The clear inter-dependence of endogenous factors, once initiated by exogenous inflammatory factors, results in the activation of the full consequence of inflammation. In the host, inflammation is likely the most important mechanism in the pathophysiology of pneumonic pasteurellosis in cattle. Further, the normal feed-forward and feed-back controls, some mediated by the beta adrenergic receptors, maybe disrupted leading to unembraced inflammation. The consequences of inflammation due to beta adrenergic receptor dysfunction may be a cause of morbidity and mortality in BRDC.

Recently the many elements of the inflammatory cascade has been tied to a central controlling factor, NFkB. NFkB is a transcription factor found in the cytosol of many cells. Once activated, NFkB, initiates multiple factors that contribute, enhance and prolong the inflammatory

process. NFkB was first described by (Sen 1986) and functions as a rapid response against environmental challenge and regulating inflammation (Baeuerle 1997).

NFkB is found in all cells. The cells that are most likely significant to the pathophysiology of BRDC are the lymphocytes (B and T cells), pulmonary alveolar macrophages (Lee 1998; Lentsch 1999; Abraham 2000) and endothelial cells (Weber 2000).

In effect, NFkB mediates a rapid mobilization of the acute inflammatory response (Abraham 2000). Endothelial cell activation of NFkB leads to activation of extrinsic tissue factor (Abraham 2000). NFkB is activated by many triggers, including LPS (Lee 1998; Abraham 2000), systemic hemorrhage (Moine 1997), hypoxia via PAMs (Lentsch 1999), leukotriene B4, thrombin, TNF-alpha, and IL-1 (Moine 1997; Abraham 2000). Each of these endogenous entities is also enhanced by the induction of NFkB and therefore constitutes a positive feedback loop (Lee 1998). This feedback can, if unchecked, create an escalating series of events leading to exuberant inflammation

The activity and activation of NFkB is well understood. A trigger, as described above, would attach to an extracellular receptor. This receptor then activates an intracellular heterodimer complex (I Kappa B, NF Kappa B). This complex then degrades, secondarily to phosphorylation, to two separate molecules I Kappa B and the activated NF Kappa B. The activated NF Kappa B then moves from the cytoplasm to enter the nucleus via nuclear pores to bind on the Kappa B binding sites of the genetic material (Matthews 1995; Baldwin 1996; Akira 1997). The binding of the NFkB dimer to the mRNA leads to an up-regulation of protein leading to increased inflammation (Lee 1998; Abraham 2000; Christman 2000; Sadikot 2000; Christman 2000A). I Kappa B acts as a concentration-dependent negative feedback system by inhibiting transcription by binding the NFkB (Arenzana-Seisdedos 1997; Tran 1997).

Proteins induced include the pro-inflammatory cytokines, chemokines (IL-8), cell adhesion molecules (ELAM-1, VCAM-1, ICAM-1), acute phase proteins (complement) oxidative stress related enzymes (COX_2, NOS, lipoxygenase) and an up-regulation of tissue factor (in endothelial cells) (Grimm 1993; Lee 1998; Abraham 2000; Christman 2000; Christman 2000A). It should be noted that TNF-alpha and IL-1 act as a positive feed back for NFkB (Blackwell 1997). This suggests one method that could lead to exuberant inflammation.

Work recently completed in human patients with Acute Respiratory Distress Syndrome (ARDS), has demonstrated an increase in activated NF Kappa B in pulmonary alveolar macrophages (Schwartz 1996). As previously noted, the PAM may also be the source of the initiation of exuberant inflammation secondary to hypoxia. Recent experiments, in humans, have supported this concept (Schwartz 1996). Hypoxia has lead to an increase in the number of macrophages and monocytes in an anaerobic micro-environment (Lewis 1999). Others have suggested that PAMs, in other respiratory diseases (e.g. asthma) in humans, may be the initiating event in ARDS (Christman 2000A). In many pulmonary diseases of man, there have been increases in the numbers of macrophages, as well as a decrease in their systemic cellular half-life, circulation time and an increase in TNF-alpha production (Van Furth 1989), TNF-alpha receptors (Ghezzi 1991; Scannell 1995; Scannell 1999), and an increase in the lymphocytes CD11b and CDe receptors (Scannell 1995). Hypoxia increases the generation of TNF-alpha within the PAM, which in a hypoxic environment there is exuberant inflammation leading to avascular necrosis (Van Furth 1989). These changes may not be seen on initial broncho-alveolar lavage in-vitro since minimal changes in PAM are expected to occur in the first 72 hours in an anaerobic environment (Cazin 1990). However changes in PAM have been shown to occur much earlier. In rabbits, there is a decrease in phagocytic activity in PAMs in hypoxia after only an hour (Leeper-Woodford 1992) . It should be noted that a minimal basal release of NFkB occurs in the PAM even in an aerobic environment (Christman 2000). This information along with an increase in PAM-NFkB and cytokine production in the face of decreasing antimicrobial activity suggests the possibility that PH-

LPS destruction is occuring while the pro-inflammatory agents increasing in the vascular micro-environment. These events are likely synergistic and may explain the ravages of the pathogenesis of pneumonic pasteurellosis without the need for significant infection. Hypoxia leads to an increase in NFkB due to an induced degradation of negative feedback molecule I Kappa B (Grimm 1993). It is well known that LPS in gram negative sepsis will activate the coagulation system (Taylor 1990). Lung injury decreases with a decrease in the number of PAMs, as well there are proportional decreases in NFkB and its products TNF-alpha, adhesion molecules, and PMN accumulation (probably secondarily to the decrease elaboration of IL-8) (Lentsch 1999). There is some discrepancy between studies with respect to the relationship between NFkB and IL-8, chemokine. Though all studies agree that NFkB can induce IL-8, the relationship may not be a directly linear ratio or may be multifactorial (Christman 2000). In fact, the best prognostic indicator of a fatal outcome in ARDS is based on NFkB binding activity in peripheral monocytes (Bohrer 1997). PGE_2 an anti-inflammatory protaglandin increases with an increase in NFkB represents a negative feedback system with respect to inflammation (Grimm 1993).

The activity of the beta adrenergic receptor on immunologically active cells including: PAMs, PMNs and lymphocytes is to depress or control inflammatory activity (Barnett 1997). The role of the beta adrenergic receptor directly with respect to NFkB has been investigated, but remains obscure with relation to pulmonary vascular disease (Baeuerle 1997). In one set of experiments, it was suggested that an increase in cAMP could result in a decrease in NFkB through a measured decrease in I kappa B the deactivating portion of the initial heterodimer. This is considered weak evidence of beta adrenergic receptor activity in the modulation of NFkB activity. There is some other circumstantial evidence for the beta adrenergic modulation of NFkB pro-inflammatory activity. A second study showed that hemorrhage and LPS result in an increase in both NFkB and cAMP in peripheral PMNs (Shenkar 1999). The source of the cAMP was presumed to be the beta adrenergic mechanism. It was shown that cAMP leads to a CREB complex that then directly connected with NFkB activation is accomplished by two different mechanisms. These results

suggest that hemorrhage activation of NF kappa B is mediated by Beta adrenergic receptor (Abraham 2000). This paper offers a suggestion that an increase in cAMP will have an inhibitory effect on the pro-inflammatory activity of NFkB. The paper by LeTulzo suggests another mechanism for the modulation of NFkB by cAMP. The action is proposed to be a decrease in NFkB activity secondary to phosphorylation of the I Kappa-b alpha to I Kappa B which, in an increased concentration, serves to reform the heterodimer with NFkB to become inactive (Ollivier 1996; Le Tuzlo 1997; Parry 1997).

The significance of the beta adrenoreceptor with respect to NFkB has yet to be evaluated with respect to the pathogenesis of BRDC. However, it has been demonstrated that thrombin can inhibit a BAR activity in PMNs leading to exuberant degranulation and this event is mediated by NF Kappa B and Il-8 (Ollivier 1996). Finally, an increase in the BARC intermediate, cAMP, has been shown to increase many adhesion molecules (ELAM-1, VCAM-1 and ICAM-1) (Ollivier 1996). This evidence is highly suggestive that the BAR is integral to the modulation of NFkB. Further, it is clear that NFkB is integral to the multi-factorial events resulting in vascular and pulmonary inflammation. It remains to be demonstrated whether disruption of the BARC by any mechanism may result in exuberant inflammation via the NFkB.

Beta receptors are clearly important in the modulation of both vascular and leukocytic homeostatic activity. There is evidence that PH endotoxin may uncouple the beta adrenergic receptor from its ability to generate cAMP via adenylyl cyclase. This uncoupling would result in a decreased receptor expression (van Heuven-Nolsen 1986; Forse 1989, Sales 1989). It has been suggested that the uncoupling, by *E.coli* endotoxin, of the cardiac beta adrenergic receptor occurs between the beta receptor and adenylylate cyclase, with no significant change in binding site affinity (Harlan 1981). The diminished cardiac response of the beta-1 cardiac adrenergic receptor is distal to the agonist-receptor interaction. Direct adenyl cyclase stimulation, by sodium floride, is unaffected by *E.coli* LPS with respect to the BAR complex (Campbell 1993). This additional

evidence supports the concept that beta adrenergic dysfunction contributes to the lack of cardiac response to beta-adrenergic agonists in endotoxemia leading to impaired myocardial function (Romano 1985; Campbell 1993; Bensard 1994). It is likely that the pulmonary vascular system may be similarly affected by endotoxin. The deficits described by Weekley (1993E) may be due to G-protein receptor deficits associated with the uncoupling of the beta adrenergic signal transduction system.

The importance of the pulmonary homeostatic beta receptors in the pathogenesis of BRDC should not be underestimated. It has been shown that in endotoxemia the beta-2 adrenergic selective agonist terbutaline can reduce microvascular serum leakage (Sigurdsson 1988). This beta-2 agonist is likely to act at all beta receptors although for the purposes of BRDC, the majority of ameliorating effects would likely occur on the endothelial based beta receptors (Steinberg 1984; Mizus 1985; Minnear 1986). Also, following the administration of terbutaline, there is a reduction in the negative hemodynamic and respiratory effects of endotoxin administered to sheep (Sigurdsson 1989). There is a similar beta adrenergic mediated anti-inflammatory effect on the macrophage cytokine product, TNF-alpha (Hu 1991; Severn 1992). This suggests that treatment of an endotoxemic animal with a beta-2 selective agonist may be beneficial in alleviating the acute symptoms and tissue pathology seen in BRDC and also associated with endotoxemia.

The importance of the beta receptor in the regulation of the homeostatic elements of the endothelium, vascular smooth muscle, platelets, neutrophils, and macrophages is well established in multiple species. The beta adrenergic receptor, though important in various pulmonary vascular homeostatic mechanisms, has largely been ignored with respect to the pathophysiology of bovine pneumonic pasteurellosis. Weekley, in the articles cited earlier, has shown that the activity of PH in-vivo can disrupt beta adrenoreceptors on vascular smooth muscle in several species. A relatively new macrolide antibiotic, tilmicosin phosphate (Micotil® 300), has been shown to have exceptional efficacy in the treatment of bovine pneumonic pasteurellosis. In preliminary, unpublished

experiments, this antibiotic also appeared to have significant beta adrenoceptor activity which may account for its clinical efficacy in cattle and toxicity in species other than the bovine.

The sum of the findings presented above is that the investigation of the beta-adrenoceptor is not only relevant to the pathogenesis of pneumonic pasteurellosis but may be a new line of investigation for the prophylactic, metaphylactic and treatment modalities for bovine respiratory disease. This research is to evaluate beta adrenoceptor dysfunction as a major mechanism in the pathogenesis of BRDC. BAR control inflammation through many redundant, multifactorial mechanisms the most important of which is NFkB. Beta receptors may be disrupted by many factors though, with respect to pneumonic pasteurellosis, thrombin and LPS appear to be the most crucial elements. The disruption of beta receptors may be the one significant factor resulting in vascular and pulmonary homeostatic disruption. It is likely that exuberant inflammation, that results in the morbidity and mortality of pneumonic pasteurellosis, culminates in the devastating economic consequences noted for this disease. The ability to understand, modulate or control inflammation through either the manipulation of the beta adrenergic receptors or NFkB is the target for the development of effective treatments, metaphylaxis and prophylaxis for BRDC.

I would propose that NFkB should be the target of treatment modalities for pneumonic pasteurellosis. Further, disruption of the BAR is one of the key element in the development of runaway inflammation and the interrelationship between NFkB and the BAR the subject of intense investigation with respect to the pathogenesis of BRDC. Many experiments demonstrate and support the prospect that the disruption of the BAR is the cornerstone of excessive inflammation involving the action of the NFkB molecule.

The advantages of an approach investigating the relationship of NFkB and the beta adrenergic receptors are clear. First, the use of antibiotics, in food animals, is currently under public scrutiny and criticism. This is particularly in the European Common Market countries, where it is

banned from use. Second, as pointed out earlier, the inflammatory process can account for more lesions related to this disease than the consequences of bacterial invasion (infective) process. To adequately mute the inflammatory process, one must recognize that much of the control in the normal animal is by way of the beta adrenergic receptor and NFkB and that this mechanism is disabled in the infected animal by the activity of endotoxin and thrombin. This dissertation investigates the hypothesis that the beta adrenergic receptor could be the primary target for the treatment for pneumonic pasteurellosis. Further, the modification or up-regulation of the NFkB system is likely to be of significant consequence in the pathogensesis of pneumonic pasterellosis.

I therefore propose, that unregulated tissue destruction, as a result of uncoupled beta adrenergic receptors, is a major factor in the pathogenesis of pneumonic pasteurellosis. Further, the activation of cellular, pulmonary and vascular beta adrenergic receptors is likely to be beneficial in the treatment and prophylactic prevention of BRDC and pneumonic pasteurellosis.

III. EXPERIMENTS COMPLETED

A). EVALUATION OF THE BETA ADRENERGIC RESPONSE OF VASCULAR SMOOTH MUSCLE FOLLOWING EXPOSURE TO *PASTEURELLA HAEMOLYTICA* A-1 IN THE RAT

Earlier studies by Weekley et al demonstrated that a disturbance in in-vitro vascular smooth muscle relaxation occurs after prophylactic vaccination with *Pasteurella haemolytica* (serotype A, biotype 1) in several species (Weekley 1991A; 1993A; 1993B). The dysfunction was described as a reduction in vascular smooth muscle relaxation as a response to the beta adrenergic agonist isoproterenol. The clinical significance and molecular mechanisms of this deficit have yet to be investigated.

The anatomy of the ruminant lung is different from that of other mammals. The bovine lung is anatomically much smaller with respect to the body mass than other animals. This suggests that the lung could be more susceptible to disease since its metabolic workload is proportionally greater than other mammalian species. In the ruminant acinus there are no pores of Kohn which, in other species, facilitate inter-alveolar communication and enhance air circulation to the vasculature (Robinson 1984; Weekley 1995; Wren 1995). Since the majority of vascular relaxation and constriction is mediated "down stream" of the post-arteriolar capillaries at the level of the micro-capillaries and post-capillary venules (Green 1982) a decrease in blood flow in combination with the isolation of the alveoli from the bronchiolar tree can result in an interstitial tissue metabolic acidosis (Green 1982). Further, a decreased flow of blood, or disruption of laminar flow results in the rolling and eventual diapedesis of leukocytes, particularly neutrophils (Hogg 1993). Beta adrenergic receptors have been demonstrated to blunt the chemiluminescent response to stimulating agents and thus may act as a "safety switch" preventing exuberant free radical and protease

liberation. If uncontrolled this may lead to extensive tissue damage (Engels 1985; Ogunbiyi 1986; Borregaard 1988; Barnard 1992; Opdahl 1993; Seccombe 1994).

The beta receptor exists in close association with the endothelium (Steinberg 1984) and is involved in the regulation and inhibition of thrombin mediated vascular permeability (Mizus 1985; Minnear 1986). Other beta adrenergic receptor mechanisms modulate the destructive action of neutrophils in immunologically mediated events (Busse 1984; Mueller 1988; Bowden 1994). Reductions in vascular perfusion, as a result of an increased vascular tone may have deleterious metabolic consequences. Disruption of the beta adrenergic mechanism, at the endothelium level, can result in increased vascular tone in an indirect manner.

Given the importance of the beta adrenergic system, in regulation of microvascular events, it would appear that disruption of this receptor system has the potential to result in severe tissue damage, enhance the disease processes and precipitate the debilitation of the animal. The dysfunction of beta adrenergic mechanisms may be an important step in this pathogenic mechanism leading to pneumonic pasteurellosis in ruminants and may also be a consequence of post vaccination exposure. The implications of the original work by Weekley (1993B), when considered with the epidemiological evidence that monovalent *Pasteurella haemolytica* A-1 (PH) based vaccines lack immunoprotective efficacy and results in decreased weight gain, (Martin 1983; Thorlarkson 1990) suggests that there may be a positive relationship between vaccination and decreased animal thriftiness. One factor in this complex equation may be the disruptive effect that some monovalent PH vaccines appear to have on beta adrenergic system on the rat aorta. Similar dysfunctional changes in response to prophylactic PH vaccination in cattle and sheep have been demonstrated following intramuscular injection (Weekley, 1993A; 1993B; 1993E).

The nature of the disruption of beta adrenergic receptor mechanisms has yet to be investigated. One may suspect that a functional disruption can occur as a single event in time

resulting in the disruption of the beta adrenergic mechanism. It is reasonable to expect that there is a minimum level of dose of *Pasteurella haemolytica* A1 necessary to induce beta adrenergic receptor system disruption.

This dissertation will describe the time course of the initiation and duration of the disruption of the beta receptor. Further investigations will assess the vaccine dose necessary to cause a pharmacologically defined disruption of the beta adrenergic receptor mechanism following the intraperitoneal injection of PH in the rat. Tissue relaxation and electron microscopic morphological changes in the vascular endothelium, in ex vivo aortic segments, will be examined with respect to vaccination kinetics and dose.

METHODS AND MATERIALS

A) Preparation of Vaccine

Pasteurella haemolytica organisms were subcultured from a commercial vaccine Precon® (A.H. Robbins Co., Richmond Virginia, lyophilized vaccine). This was accomplished by growth of the initial vaccine in broth overnight in a sterile Erlenmeyer flask (1liter calf brain, heart infusion broth [BHI] in an incubator at 37^0 C with gentle swirling in an incubated orbital shaker for 12 hours). An aliquot of fifty milliliters of the resulting broth culture was then removed and transferred to a sterile Erlenmeyer flask containing 500 milliliter volume of BHI, and incubated at 37^0C with gentle swirling. This second broth culture was then evaluated for turbidity representing bacterial growth, using a Klett meter every sixty minutes until there was no change between two consecutive readings. At each Klett meter reading, an aliquot of the sample (500 microliters) was obtained using sterile technique and the number of colony forming units (CFU / milliliter) was determined by a serial dilution and plating of each dilution. The final product was then centrifuged at 3000 x g for 15 minutes and the supernatant decanted and discarded. The remaining organisms

were then resuspended in a BHI aliquot of 1 milliliter in a polyethylene tube and frozen at -70^{0}C until needed. Twenty-four hours before vaccination, one aliquot of the prepared vaccine was thawed to room temperature over several hours first on ice then in cold tap water. The vial was then disinfected by the external spraying of 70% ethanol and placed in a laminar airflow biohazard hood. Once in the biohazard hood aseptic conditions were maintained throughout all subsequent procedures. Serial logarithmic dilutions were made of the aliquot and plated on bovine blood agar (blood was obtained from PH naive animals) and evaluated for the number of colony forming units of PH. This procedure allowed the assessment of the number of colony forming units per milliliter (CFU/ml). This allowed the appropriate dilution of the vaccination dose by sterile normal saline. Animals were injected with the appropriate dose of bacteria administered intraperitoneally. The injected volume never exceeded 1.0 ml. Randomly, re-evaluation of the number of colony forming units (CFU) per milliliter was calculated from the aliquot remaining after intraperitoneal injection. In all cases the CFU's assessed before vaccination were with-in 10% of those calculated post-vaccination.

B) Preparation of Drugs and Solutions

Krebs-Henseleit buffer was used in the biophysical evaluation and dissection of the ex-vivo aortic rings. The composition of the Krebs-Henseleit solution (in one liter of sterile double distilled water in a sterile 5 liter Erlenmeyer flask as follows: 6.9 grams NaCl ; 0.35 grams KCl; 0.28 CaCl; 0.14 grams $MgSO_4$; 2.09 grams $NaHCO_3$; 0.16 grams KH_2PO_4; 2.0 grams dextrose as described by Weekley (1994). This solution was aseptically mixed immediately before use.

Potassium chloride was used for initial smooth muscle contraction. This stock solution was prepared in sterile, double distilled water to ensure a 30 millimolar concentration when 200 microliters of stock KCl solution was added to 30 milliliters of the Krebs Henseleit bathing solution.

Isoproterenol, a beta adrenergic receptor agonist, was used to evaluate the beta adrenergic mechanism of isolated, ex-vivo, aortic vascular rings. This solution was prepared in sterile, double distilled water. Serial decreasing logarithmic concentrations of isoproterenol were made from the initial 10^{-2} molar solution. The serial dilutions were made by taking 100 microliters of the initial stock solution and diluting this in 900 microliters of sterile double distilled water sequentially to 10^{-9} molar solution.

C) Treatments and Subjects

Adult male Sprague-Dawley rats (Harlan Sprague-Dawley Inc., Indianapolis, Indiana) approximately 150-175 g, on arrival, were used in this series of experiments. The animals initially were housed in a quarantine area for a one week for acclimatization to the new facility. All animals were examined physically for external signs of disease. Any animals showing any external signs of disease were eliminated from this study. The acclimatization period involved a 12:12 light dark photo period, room temperature was 22^0 +/- 3^0 C.

I) Examination of the time course of beta adrenergic receptor disruption

Pasteurella haemolytica biotype A serotype 1, obtained from Precon® live lyophilized vaccine, at a concentration of 10^5 CFU's, was injected intraperitoneally into subjects. Concentration of bacteria in each injection was evaluated by serial logarithmic dilution of previously grown and frozen (-70 C^0) aliquots of PH. After injection random aliquots were re-evaluated to assure that there were approximately 10^5 CFU in the injection solution.

Forty rats were vaccinated on a single day over a period of one hour. Each animal was assigned to one of ten experimental groups (n=4) identified as "Day" 0, 1, 2, 3, 4, 5, 6, 7, 14, or 21.

Day 1 represents the first day post vaccination (twenty-four hours), day 2 represents the second day post vaccination and so on through Day 21. Both negative (day 0, no exposure to PH) and positive controls (day 3, post-exposure to PH at concentration of 10^5 CFU) were obtained according to the procedure described by Weekley and Eyre (1993B)

II) Examination of the dose of *Pasteurella haemolytica* necessary to initiate beta adrenergic receptor dysfunction

Six experimental groups were formed by assigning animals (n=4) to a group. Each group was exposed to one experimental treatment . Serial logarithmic concentrations of PH (from 0 CFU (negative control) through 10^5 CFU (positive control) yielded the six experimental treatments (0 CFU, 10^1 CFU, 10^2 CFU, 10^3 CFU, 10^4 CFU, 10^5 CFU) used in this experiment. *Pasteurella haemolytica* biotype A serotype 1 at a concentration of 10^5 CFU's was serially diluted to give the appropriate concentration of colony forming units to be injected intraperitoneally into rats. All groups were evaluated at 72 hours post parenteral exposure to PH.

D) Tissue Collection and Measurement Techniques

Rats were anesthetized with sodium pentobarbital (80 mg/kg) immediately followed by a separate administration of 25 U of sodium heparin intraperitoneally. Once a deep surgical plane of anesthesia was reached the abdomen was opened to reveal the diaphragm. The approach to the thorax and thoracic aorta was through the diaphragm. The ventrum of the thorax was removed to completely expose the thoracic contents. Once exposed, the aorta from the base of the heart to the last rib was carefully removed ensuring that no damage or stretching occurred. A segment of the aorta, from the base of the heart to the distal portion of the thoracic limb, was then placed in warm aerated Krebs-Henseleit (37^0+/- 2°C, 95% oxygen: 5% carbon dioxide) for further dissection and removal of extraneous fat and other superficial tissue. All aortic rings were obtained from the base

of the heart. The rings were carefully measured to be 3 mm in length. Exceptional care was taken to ensure that there was no stretching or damage to the endothelium. The segment was then used for biophysical testing. A second segment immediately adjacent to the 3 mm segment used for pharmacological testing was then removed from each aorta for examination by electron microscopy.

The 3 mm aortic ring removed for the biophysical testing was then mounted between two glass hooks. Biophysical testing involved the assessment of the change in tension (isometric contraction) of the aortic ring due to the addition of known concentrations of various drugs. The ring was placed between the two hooks in a thirty-five milliliter tissue bath containing Krebs-Henseleit bicarbonate buffer. One hook was attached to a fixed point at the bottom of the tissue chamber while the other hook was attached to an electronic transducer that was calibrated to give output in grams tension. The tissue was placed in an insulated heated tissue bath aerated with 95% oxygen and 5% carbon dioxide through each of four individually isolated tissue baths. A constant tissue bath temperature of 37.0^{o}C (+/-2^{o}C) was maintained by circulating water through a temperature controlled water pump and sequentially through the outer heating jacket of each tissue bath. Each tissue ring was subjected to 2 grams resting tension for two hours and allowed to equilibrate. The Krebs-Henseleit buffer was replaced with fresh buffer every 15-20 minutes throughout equilibration and between testing sessions. Testing started immediately after the equilibration period. The aortic rings, in the tissue bath, were contracted with 200 μl of potassium chloride (KCl, 30 millimolar) and allowed to equilibrate to a stable level over 30 minutes. After equilibration to the contraction effects of the KCl, sequential increasing log molar doses of isoproterenol were added.

Electronically calibrated analog signals were transmitted from the transducer connected to the glass hook through an analog digital converter (MacLab A/D signal converter, World Precision Instruments). Digitized information was captured by MacLab data acquisition software (MacLab 2.51) on a Model M5011 Macintosh SE computer (1MB RAM, 800 K drive, 20 sc hard disk) in

real time. Records of vascular smooth muscle activity, as a cumulative response to log molar increases in isoproterenol were obtained and the results stored on a 3.5 inch computer disk.

E) Scanning Electron Microscopy

The sections of aorta harvested for electron microscopic examination were immediately placed in a solution of 5% gluteraldehyde and Krebs-Henseleit solution. Once fixed, (minimum time 7 days) the tissue was washed with 0.1 mol/L sodium cacodylate buffer (pH 7.3) then dehydrated through a series of increasing concentrations of ethanol. Tissue was then freeze dried using liquid carbon dioxide and a vacuum, mounted on aluminum stubs, sputter coated with gold in such a manner that the aortic luminal surface was exposed for examination. Electron microscopic examination was completed using a JOEL 100 scanning electron microscope.

These sections were examined for any changes indicating endothelial structural abnormality for both the kinetic and dosage experiments. Each tissue was examined for any signs of damage as a result of dissection, handling or the experimental glass hooks. In no case were any signs of damage due to the handling techniques observed. Photographs were made of samples that were representative of the experimental group (n=4) from which they came. All photographs are identified by numbers, referenced to the original experimental group and have 10 micrometer reference bars for ease of comparison.

F) Statistical Analysis of Data

Data were analyzed in several ways. Initially the data were compared for each experimental treatment at the isoproterenol concentration of 10^{-5} molar. In pilot studies this concentration of isoproterenol resulted in a data point in the middle of the generated dose response curve and so was selected to be an appropriate point for comparison of different groups. The comparison was done

by use of an analysis of variance (ANOVA) using group (n=4) means and standard error of the mean. This comparison was done to evaluate if there was a significant change in aortic ring tone at 10^{-5} molar isoproterenol among treatment groups. Comparison of the values at 0 molar isoproterenol and 10^{-5} molar isoproterenol were made to assess the amount of relaxation from the initial baseline for each treatment. These comparisons were accomplished by use of Student "t" test. This comparison was to evaluate if there was a significant change with increasing logarithmic doses of isoproterenol.

RESULTS

A) Examination of the time course of beta adrenergic receptor disfunction

The time course of the response of vascular smooth muscle to beta adrenergic stimulation is complex over increasing doses of isoproterenol (figure #1). Within group variation for overall group responses, was assessed by comparing the change in tension (relaxation or constriction) at the two extremes of the dose range for isoproterenol (0 molar, baseline and 10^{-5} molar). This comparison was done by use of a Student "t" test. The negative control (day-0) demonstrates the classical sigmoid curve for beta adrenergic mediated vascular smooth muscle (figure #1) response. In this case, relaxation was observed and was found to be significantly different ($P< 0.0001$) from the baseline. The positive control (day-3) indicated no relaxation in response to isoproterenol mediated beta adrenergic stimulation. This confirms the findings of Weekley et al. (1993A, 1993B and 1993E) in previous experiments. The lack of response to isoproterenol also occurred at day 21 between the two extremes of isoproterenol concentration. For days 1, 2, 4, 5, 6, 7, and 14, there was no apparent isoproterenol mediated relaxation. Further, each of these days demonstrated a significant increase in tension at 10^{-3} molar isoproterenol when compared to baseline ($p< 0.05$). On day 1, there was a trend toward relaxation between the molar isoproterenol concentrations of 10^{-7} M and 10^{-5} M. Day one demonstrated relaxation to the isoproterenol stimulation, as the negative

control, however there was a significant increase in tension, reversal of potency ($p < 0.0001$) noted above 10^{-5} molar isoproterenol.

Using an ANOVA it was determined that the variance among the tissue tension means (at 10^{-5} M isoproterenol) was significantly greater than expected by chance ($p < 0.0001$). Pairwise comparisons by the Student "t" test. for each group mean (n=4) indicated a significant difference between day 0 (negative control) and all other groups ($p < 0.001$) except day 1 where the difference was insignificant ($p > 0.05$, figure #2). Day 1 was significantly different from all other groups, except day 0.

B) Examination of the concentration of *Pasteurella haemolytica* AI on beta adrenergic system disruption

A two way ANOVA was performed on the isoproterenol dose on the experimental data from each PH dose group. Statistical, within groups, comparison of the extent of relaxation over the range of concentrations of isoproterenol was completed (figure #3). The negative control (0 CFU PH) showed significant relaxation ($p < 0.0001$) as compared to all other experimental conditions at 10^{-5} molar isoproterenol. There was also a significant increase ($p < 0.001$) in contraction of the group of experimental tissues (10^1 CFU to 10^4 CFU) beyond the potassium chloride pre-contraction (baseline) condition. Finally, there were no significant differences in baseline tension ($p > 0.05$) for the 10^5 CFU (positive control) experimental tissues versus the other exprimental treatment conditions (10^1 CFU through 10^4 CFU). A graphical description of experimental data across the increasing logarithmic concentrations of isoproterenol is presented in figure #3.

A one way analysis of variance (ANOVA) of groups at the 10^{-5} molar isoproterenol concentration determined that the variance among the means was significantly greater than expected

by chance ($p < 0.0001$). The means were then compared in a pair-wise fashion using the Student-Newman-Keuls multiple comparisons test. The negative control (0 CFU) was significantly different from all other groups ($p < 0.001$) and demonstrated a relatively complete relaxation. The positive control (10^5 CFU) was not significantly different from groups 10^2 CFU to 10^4 CFU. The group exposed to the concentration 10^1 CFU was also found to be intermediate to the negative control and all other groups. Though not statistically significant there was a steady progressive increase in the inhibition of relaxation to a maximum at 10^4 CFU. The positive control (10^5 CFU) showed a relative decrease in the inhibition of the beta adrenergic mediated smooth muscle response to isoproterenol with respect to groups 10^2 CFU to 10^4 CFU (figure #4).

C) Examination of the morphological changes associated with the time from exposure and the dose of the exposure

Tissues collected for each subject were examined. The luminal endothelium of each tissue was observed under a scanning electron microscope. After microscopic examination, each tissue in a group (n=4) a representative section of the endothelium was identified and photographed. After all tissues had been examined the photographs were then reviewed for trends or patterns that might parallel treatment levels.

Examination of the photographs (figures #5-14) with respect to increasing time from exposure to *Pasteurella haemolytica* A1 appears to indicate increasing fibrous elements on the endothelial surface that is consistent with fibrin. Further, on day 7 it appears as though there are vesicles associated with the endothelial surface as described by Weekley (1993A). These ulcerations / vesicles have been associated in time with the disruption of the beta adrenergic mediated smooth muscle relaxation. The photo-micrographs are presented below (figures #5-20).

Figure #1: Comparison of data over time for PH at 10E5 CFU.

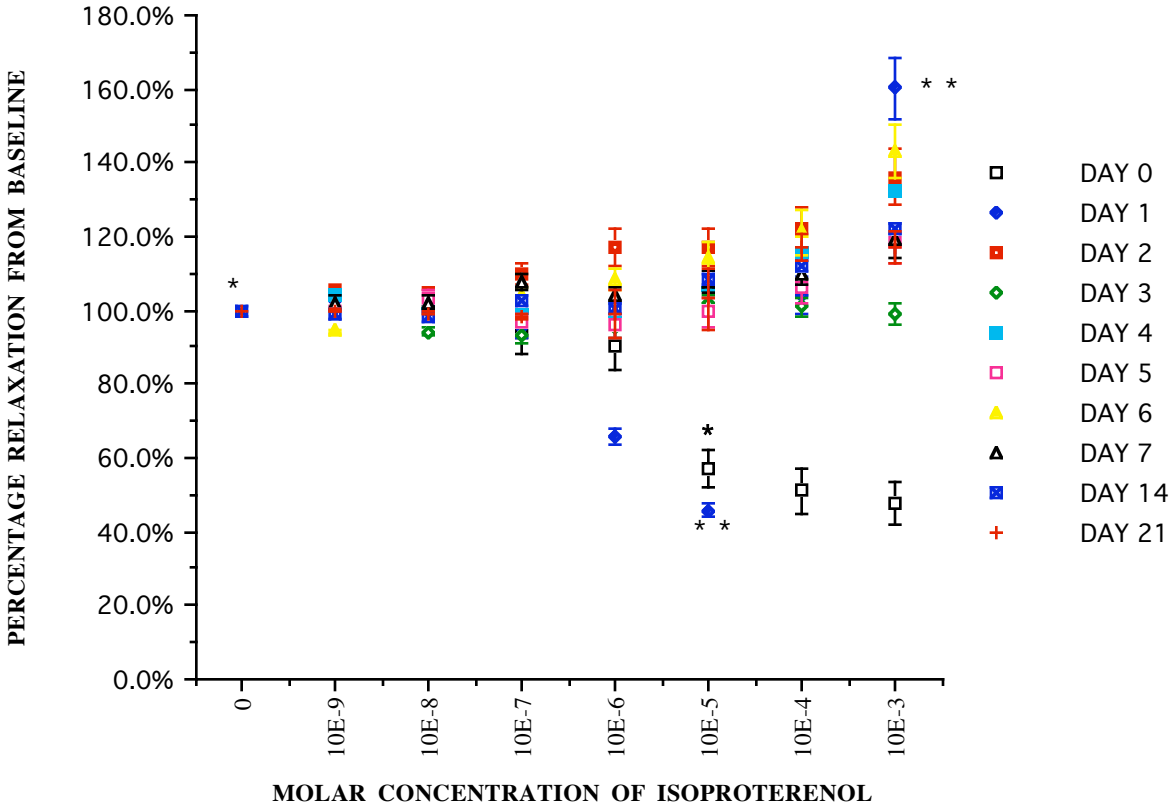


Figure #1.1: The effect of increasing molar isoproterenol activation on beta adrenergic receptor complex mediated vascular smooth muscle relaxation of rat aorta. Subjects (n=4) were given PH (10^5 CFU-IP) before testing (day 0 to day 21 as noted). All data were reported as Mean (+/- SEM) for each day over all concentrations of isoproterenol are graphed. Significant differences ($p < 0.0001$) for relaxation (*) and reversal of relaxation ($p < 0.0001$) (**) have been indicated.

Figure #2: Comparison of mean data at 10E-5 molar isoproterenol.

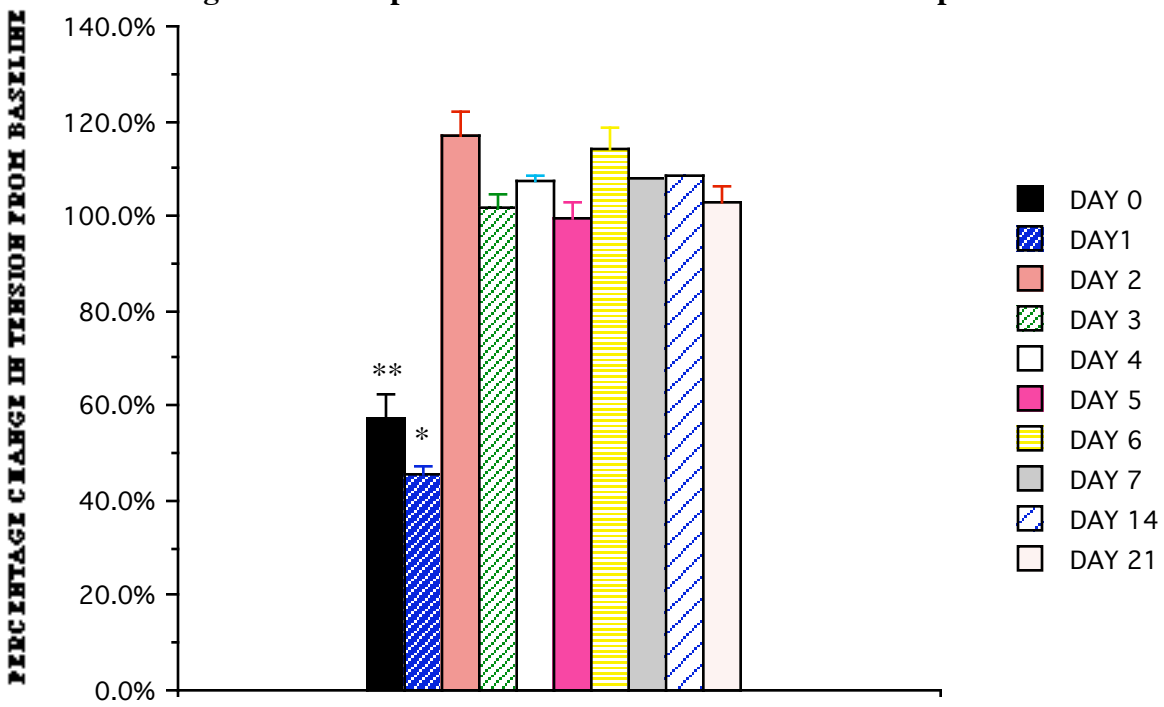


Figure #1.2: The effect of 10^{-5} molar isoproterenol on beta adrenergic receptor complex mediated vascular smooth muscle relaxation of rat aorta. Subjects (n=4) were given PH (10^5 CFU-IP) before testing (day 0 to day 21 as noted). There is a sigificant difference ($p< 0.0001$) for day 0 (**, negative control) and day 1 (*) compared to all other days. There was no significant difference ($p> 0.05$) between day 0 (**) and day 1 (*).

Figure #3: Comparison of data over all concentrations of PH.

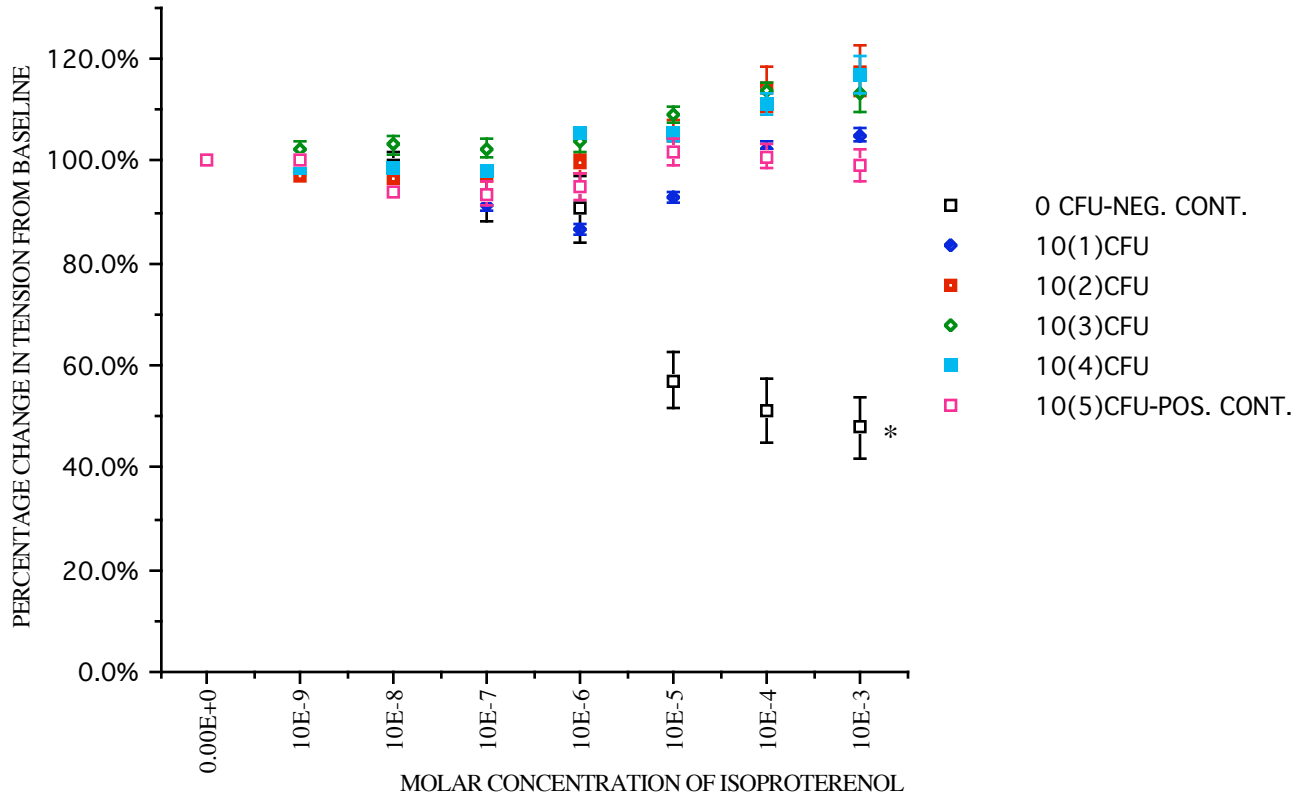


Figure #1.3: The effect of increasing molar isoproterenol activation of beta adrenergic receptor mediated vascular smooth muscle relaxation of rat aorta. Subjects (n=4) were examined on day 3 post-treatment (IP injection) over all concentrations of*Pasteurella haemolytica* (0 CFU to 10^5 CFU). All data are reported as Means (+/- SEM) for each concentration. Significant differences ($p< 0.0001$) exist between the negative control (0 CFU) and all other treatments in relaxation over all concentrations of isoproterenol (*). There wer no significant differences ($p>0.05$) among the experimental treatments (10^1 CFU to 10^5 CFU) in response tc increasing molar isoproterenol.

Figure #4: Comparison of mean data at 10E-5 molar isoproterenol.

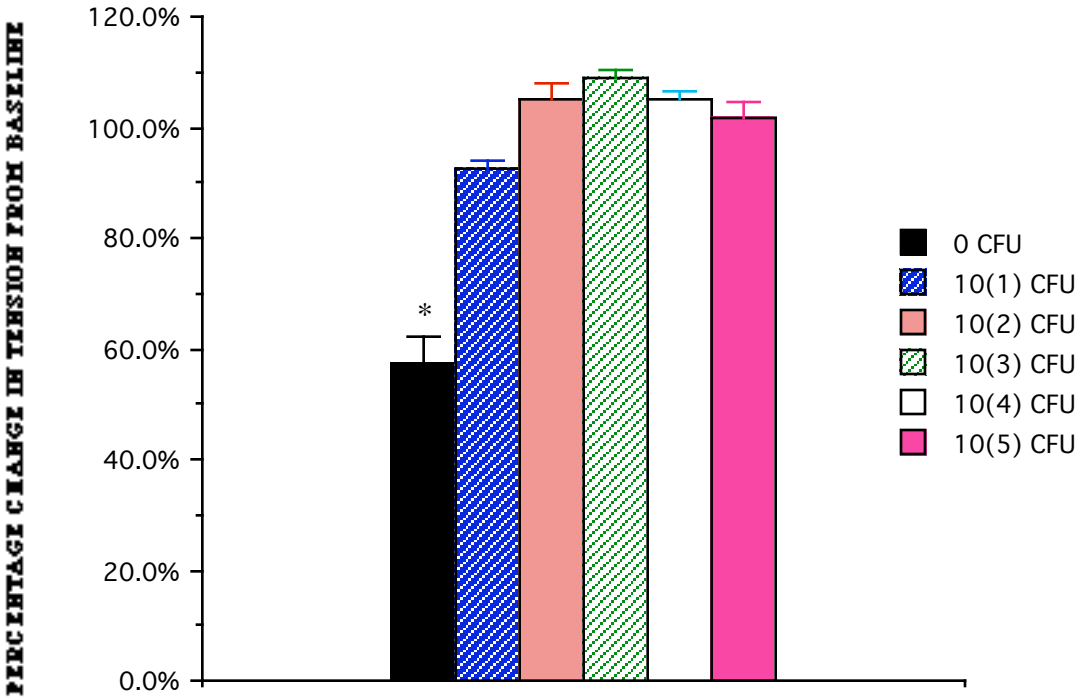


Figure #1.4: The effect of isoproterenol (at 10^{-5} molar) on beta adrenergic receptor mediated relaxation in the rat aorta. Subjects (n=4) were examined on day 3 post-treatment (IP injection) over all concentrations of *Pasteurella haemolytica* (0 CFU to 10^5 CFU). All data are reported as Means (+/- SEM) for each concentration. There was a significant relaxation ($p< 0.001$,*) noted between the negative control tissues (0 CFU) and all other experimental treatments.

Similar examination of scanning electron micrographs for the experiment involving increasing doses of *Pasteurella haemolytica* A1 were reviewed These photographs showed increasing amount of the cellular elements and fibrin-like material on the vascular luminal endothelium with increasing concentrations of *Pasteurella haemolytica*.(see figure #16-21).

DISCUSSION

A) Time Course of Beta Adrenergic Receptor System Dysfunction

Vascular adrenergic smooth muscle tone is not likely the product of a single receptor type or single drug class. All adrenergic receptors interact to result in the final vascular smooth muscle response that is measured. It is likely that the predominant initiators of relaxation of vascular tone are the beta adrenergic receptors which are activated by isoproterenol (a synthetic mixed beta adrenergic agonist). The predominant receptor initiating vascular constriction is the alpha-1 receptor. Finally, alpha-2 receptors appear to cause constriction in low vascular tone but initiate relaxation during high vascular tone (results not presented here). Further to confound this issue, the pharmacological agonists available are selective, not specific for each adrenergic receptor. This suggests that these agonistic adrenergic drugs may have interactions with other than their specific adrenergic receptors at higher pharmacological concentrations.

Comparison of these data to those of previous researchers, reveals the same pattern as that described by Weekley for the negative and positive control groups (Weekley 1993E)**.** After day 2, the loss of the smooth muscle relaxation response to isoproterenol cannot be denied. The negative control aortic rings demonstrated a typical relaxation throughout increasing concentrations of isoproterenol. The data on day 1 (figures #1 and #2) demonstrate a partial relaxation and then a reversal of the relaxation (reversal of potency) to a contraction at the higher concentrations of isoproterenol. This evidence suggests that the nature of the disruption of the beta adrenergic

mechanism the disruption of the pharmacologically mediated smooth muscle relaxation response occurs over 24-48 hours. This data of reversal of potency suggests that disruption of the adrenergic mechanism is not complete but dose dependent or related to receptor recruitment and may be overcome by increasing pharmacological doses.

The relaxation seen on day one offers several possible explanations. Endotoxin, one of the virulence factors of PH has been known to affect levels of cyclic adenosine monophosphate (cAMP). On day one, the effect of the treatment caused an enhanced, (possibly supersensitivity) response in the beta receptors which subsequently fail to respond by day 2. This is a previously reported phenomenon and is seen in denervation super-reactivity in whole animal preparations. However, a review of the literature fails to describe this event in beta receptors. Another explanation may be that the early actions of the vaccine primarily affect the adrenergic receptors responsible for vascular contraction (alpha-1). Early temporary reduction of the tonic activity of alpha one receptors would result in the data obtained for day 1 at these lower isoproterenol concentrations.

The first time point of maximum contraction of vascular tissue occurs two days after parenteral exposure to PH . This indicates that previous experiments, evaluated at three days, have obtained the expected results but have yet to characterize the maximal effect of inhibition of relaxation of vascular tissue. There are relative, though not statistically significant, increases in the amount of inhibition of the smooth muscle relaxation response at day 2 and day 7 (see figure #2). The relaxation response may be related to fluctuations in the disruption of beta adrenergic system to exposure to PH.

At the higher doses of isoproterenol there is a tendency for the tissues to demonstrate an increased constriction. At higher doses of isoproterenol (10^{-4} and 10^{-3} molar concentration) the selectivity for beta receptors may be generalized to other receptors including the alpha-1 receptor. This generalization of isoproterenol may result in an increase in alpha-1 activity and an immediate

contraction response. Similar results can be noted for all days except the negative and positive controls.

The question of the duration of the disruption of the beta receptor has been investigated. Cursory evaluation of the data may suggest a tendency to recovery from days 2 to 5 (figure #2). This trend reverses on day 6 with increasing levels of beta adrenergic receptor dysfunction to day 21. Decreased vascular relaxation after the parenteral administration of PH may occur for several reasons. If the beta adrenergic receptor protein is rendered non-functional, return to function may require the regeneration of entirely new BARCs. If the disruption is beyond the receptor protein, in the receptor mechanism cascade, the disruption may require regeneration of the appropriate molecules for full functioning. Several steps in the biochemical cascade may be particularly susceptible to *Pasteurella haemolytica* disruption. The disruption of the post-beta adrenergic receptor mechanism, the G protein, could result in failure of vasorelaxation. The disruption of the secondary messenger system, downstream of the G-protein, involving adenylylate cyclase and cAMP may be interrupted and therefore hinder vascular relaxation. There is some evidence that *Pasteurella haemolytica* can have deleterious effects on cAMP. The post cAMP mechanisms are only beginning to be investigated. The possibility exists that there is a disruption of the steps involving the myosin light chain kinase, the calcium (another second messenger) or the mechanism of the vascular smooth muscle relaxation proper. At this juncture none of these possibilities have been investigated with respect to the parenteral exposure to PH.

B) Dose of bacteria to initiate beta adrenergic receptor disruption

This series of experiments suggest that disruption is not an all or none phenomenon. The first dose increment (10^1 CFUs) reveals a statistically significant initial relaxation followed by a sudden reversal of the relaxation resulting in a final overall contraction at and above 10^{-5} molar concentration of isoproterenol. This data suggests a partial disruption of the beta adrenergic

receptor mechanism. All other results (10^2 to 10^5 CFU) indicate no significant relaxation at 10^{-5} molar isoproterenol. In fact there is a significant contraction ($P< 0.01$) for all groups, except the negative and positive controls at and above 10^{-5} molar isoproterenol concentration.

C) Examination of the morphological changes with respect to time

The morphological characteristics of the experimental treatment are more clear cut. The negative control shows no evidence of fibrin formation. The first observation of an increase in cellular elements is evident on Day 1. The thesis of this paper is that the beta adrenergic system is rendered dysfunctional after parenteral exposure to PH. The beta receptor has also been demonstrated to exist in close association with the cells of the endothelium (Steinberg 1984) and is involved in the regulation of vascular permeability, the inhibition of thrombin mediated vascular and the permeability to albumin (Mizus 1985; Minnear 1986). The inhibition of the beta adrenergic mechanisms would therefore likely result in the disruption of the control of vascular permeability and increases in thrombogenic events on the endothelial surface. Examination of the endothelial surface reveals an increase in linear "fibrin like" strands. These events appear to increase with increasing time. The events can be explained by the disruption of endothelial based beta adrenergic receptors that inhibit thrombogenic events intravascularly. The fibrin-like material on day 6 has a multi-stranded appearance and represents an increase in fiber diameter. The fibrin strands decrease significantly at days 14 and are not visible by day 21. These data appear to support the thesis that the beta adrenergic receptors are disrupted by parenteral exposure to *Pasteurella haemolytica.*

On day 7 there appeared to be a "delamination" or "vesicular formation" of the luminal surface and an apparent exposure of subendothelial tissues of the aorta. These "vesicles" were also noted by Weekley et al (1993A) The vesicles are of unknown origin however similar lesions have been noted in the pumonary vasculature after infection with *Haemophilus somnus* in cattle (Griffin, 1996) and have also been noted on endothelial surfaces of animals that have not been exposed to

PH. It is therefore possible that these "vesicles or blebs" are non-specific responses to either immunological, endotoxin or stressor challenges. A possible scenario for their origin is that unregulated beta receptors on the surface of the endothelium fail to prevent cellular elements, particularly neutrophils from interacting with the endothelial surface. As has been noted the beta receptor of the neutrophil acts to reduce extraneous and exuberant release of oxidative enzymes. The disruption of the beta receptors on the neutrophil would therefore be unable to prevent the exuberant release of the oxidative enzymes by the neutrophil. Oxidative enzymes released even in limited quantities should result in endothelial cell damage which may appear as vesicles. There is some support in the literature for this position as it has been demonstrated that neutrophil deficient animals appear to demonstrate less gross and microscopic pathological damage than animals with normal levels of neutrophils (Breider, 1991).

The evaluation of morphological events over time appears to involve the development of fibrin like strands increasing in density from day 1 through day 7. Day 7 appears to be the first day where blebbing, or disruption of the endothelial surface is noted. By day 14 the fibrin strands appear to be decreased in number and by day 21 are non-existent. Overall there is an increase in cellularity from day 1 through day 6. The endothelium appears to swell decreasing the topographical features as time progresses. A thrombogenic event is likely to follow a sequential pattern of increasing fibrin and cellularity intravascularly. These events may be followed by elaboration and release of intracellular substances that can result in delamination of the endothelial surface resulting in the blebbing noted. These results support the hypothesis that fibrin is a consequence of the initiation of throbo-embolic/endothelial activity. The blebbing is likely a consequence of increased cellularity, increased expression of ICAM, ELAM receptors resulting in the vesicle formation, delamination or blebbing.

D) Examination of the morphological changes with respect to dose of PH

There is an increase in cellularity visible on the ScEM's from 10^1 CFU to 10^4 CFU. Further, the fibrin-like strands appear to increase with increasing dose of PH. This suggests that increasing doses of PH can enhance the endothelial morphological changes seen after exposure to PH. This was not the case with the biophysical results. In a separate experiment, with respect to the morphological changes it appears that there is a threshold dose of 10 colony forming units (CFU) of PH that is necessary to initiate the events noted. The measurement of colony forming units only gives an estimate of the viable organisms. It is possible that the endotoxin level is significantly higher than is represented by the number of viable organisms. This may suggest that the morphological events seen are related to the endotoxin burden and not directly to the viable organisms.

Disruption of the endothelial based beta adrenergic receptors that inhibit thrombogenic events could lead to the initiation of fibrin formation. This is what is seen intravascularly after parenteral exposures to PH above 10^1 CFU. The morphological changes appear to support the thesis that, at the very least, one step of the beta receptor mechanism is disrupted following exposure to the PH organism which is a necessary element in this scenario.

These findings support the assertion that the potential of unregulated destruction, as a result of uncoupled beta adrenergic receptors, may be a basis for the pathogenesis of pneumonic pasteurellosis. Further, the activation of cellular, pulmonary and vascular beta adrenergic receptors are likely to be beneficial in the treatment and prophylactic prevention of BRDC and pneumonic pasteurellosis.

Figures # 1.5 - 1.13: Scanning electron micrographs (SEM) of the luminal aspect of the aorta of the experimental Sprague-Dawley rats. Tissue sections represented are adjacent to those obtained for biophysical evaluation. Figure # 1.5 represents the negative control (saline), figure # 1.6 represents the positive control (10^5 CFU-PH). Figures # 1.8 – 1.13 represent various experimental treatments as noted.

Figure #1.5: This is a micrograph of the luminal surface of the proximal aorta following antemortem IP injection of sterile saline (day 3). There are minor visible discontinuities of the luminal surface, fibrillar strands and cellular elements (consistent with fibrocytes) homogeneously cover the surface. The endothelial topography is evenly colored (gray) throughout the micrograph. There are no signs of endothelial disruption, thrombocyte or leukocyte disruption.

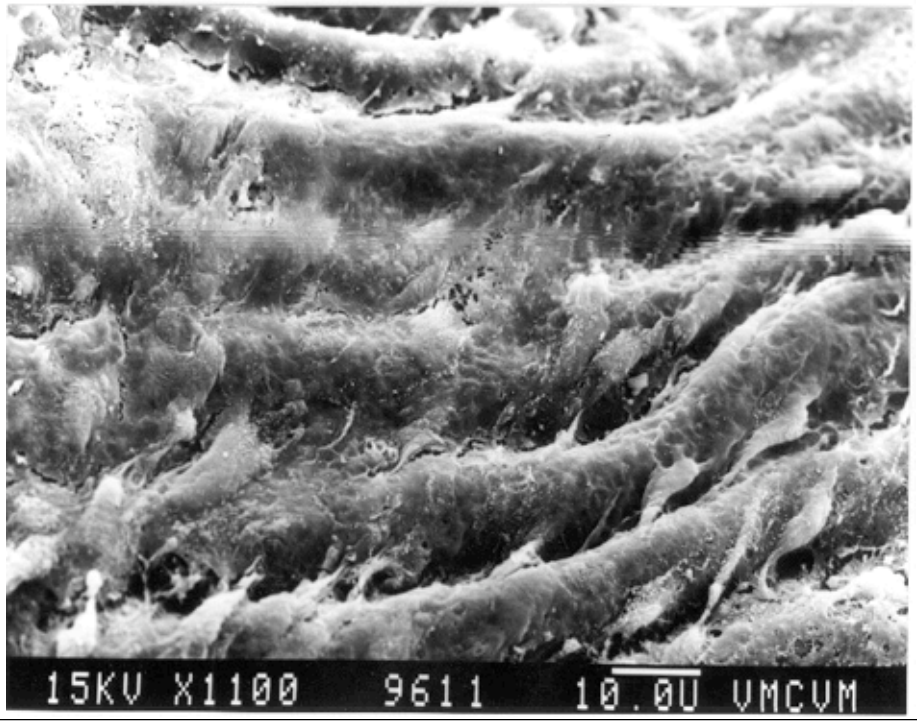


Figure #1.5: Negative Control (0 CFU PH, @ 3 days post-injection)

Figure # 1.6: This is a micrograph of the luminal surface of the proximal aorta following antemortem IP (3 day) injection of 10^5 CFU PH. There are significant disruptions of the endothelial surface at 11 and 12 o'clock positions. There are circular / spherical cell-like adhesions consistent in size and shape to thrombocytes at 1-2 and 7-8 o'clock positions. Other cell like adhesion are seen at 6-9 and 3 o'clock positions, these are consistent with leukocytes. Flattened cell like adhesions with fibrillar-like attachments are consistent with fibrocytes. The endothelial topography is not as smooth as the negative control. There are increased depth and size of rugae (endothelial folds).

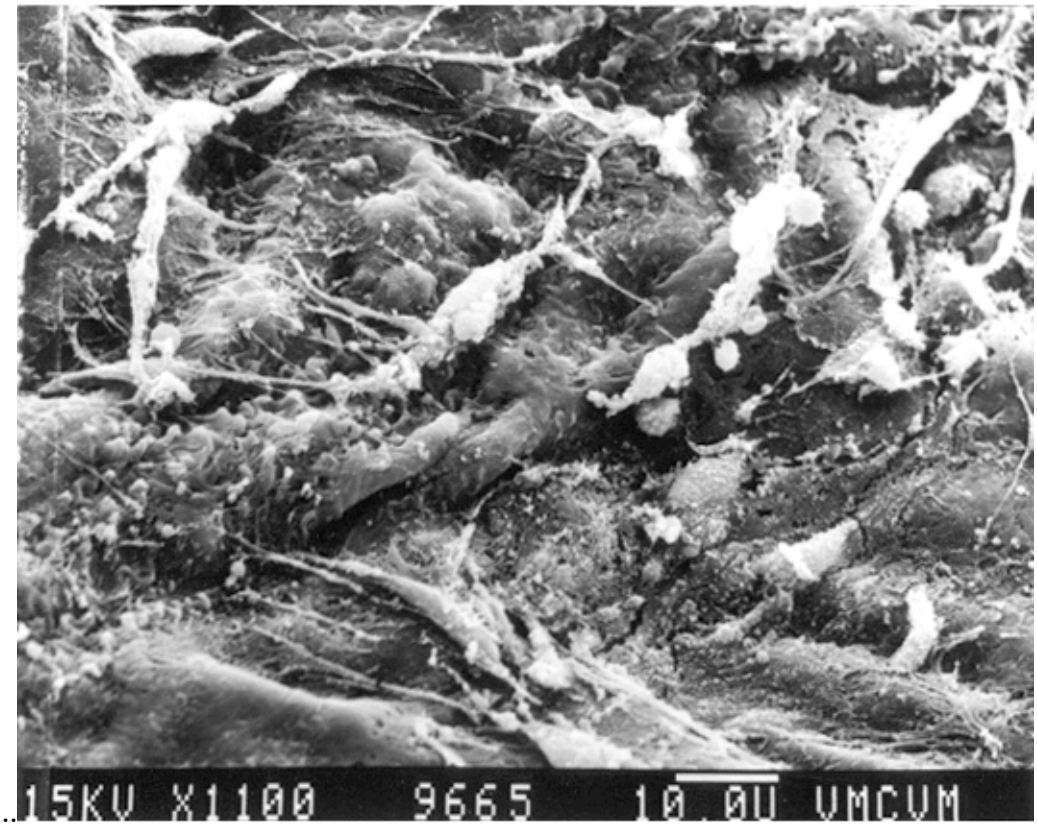


Figure # 1.6: Positive control (10^5 CFU PH, @ 3 days post-injection)

Figure # 1.7: This micrograph represents a 24 hour post injection (10^5 CFU PH) section of the proximal luminal aorta Initial changes noted in this micrograph are increased fibrillar attachment to the luminal surface, attachment of several biconcave cell-like discs (consistent with RBC's) and multiple "sphere-like" adhesions (consistent with thrombocytes). There appears to be an increase in fibrillar cellular activity (consistent with increased fibrocyte activity) at the endothelial / luminal surface.

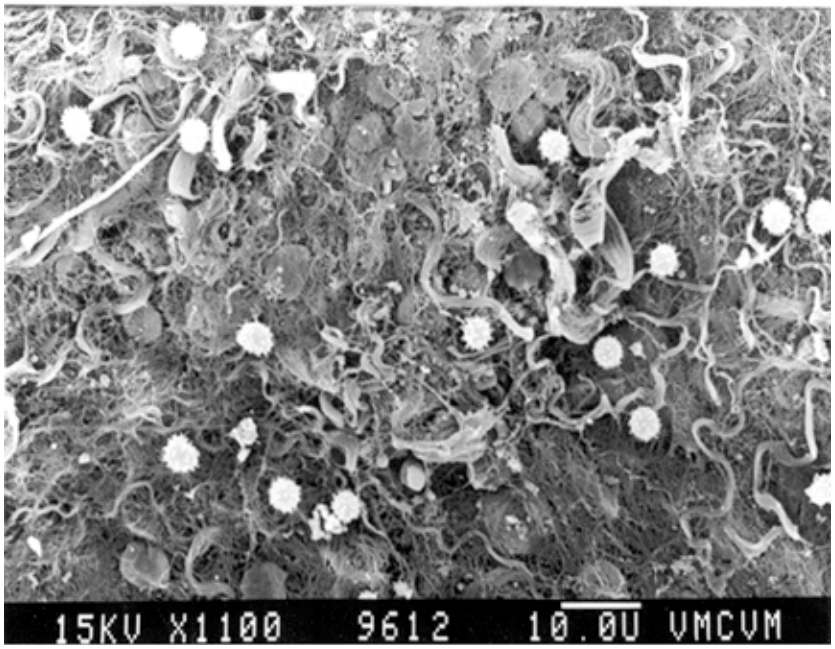


Figure # 1.7: Experimental day #1.

Figure # 1.8: This micrograph at 48 hours post injection (10^5 CFU PH, IP) shows a continuation of the increased fibrillar activity over the luminal surface noted previously (figure # 1.7). The cell-like elements (consistent with leukoctyes) appear creanated secondary to preparation artifacts. Fibrillar elements are increased over previous days. There are endothelial discontinuities creating a moth-eaten appearance to the topography. The lesions at 1, 3 and 7 o'clock positions suggest endothelial ulceration or vesicular formation.

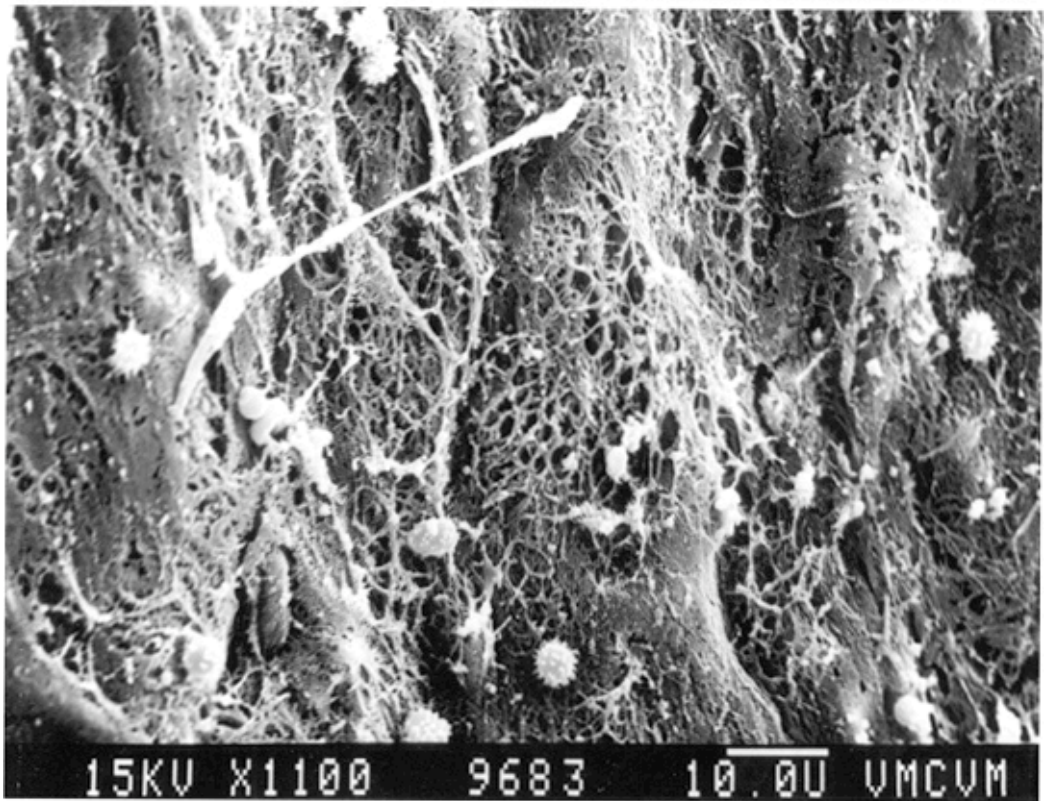


Figure # 1.8: Experimental day #2

Figure # 1.9: Day 4 following antemortem injection (10^5-CFU PH, IP) demonstrates significant disruption of the endothelial surface at the 12 and 1 o'clock positions. The ulcerated areas of the endothelial surface are consistant with disrupted bullae or ulceration's. Fibrillar elements are consistant with those noted in the 2 and 3 day micrographs.

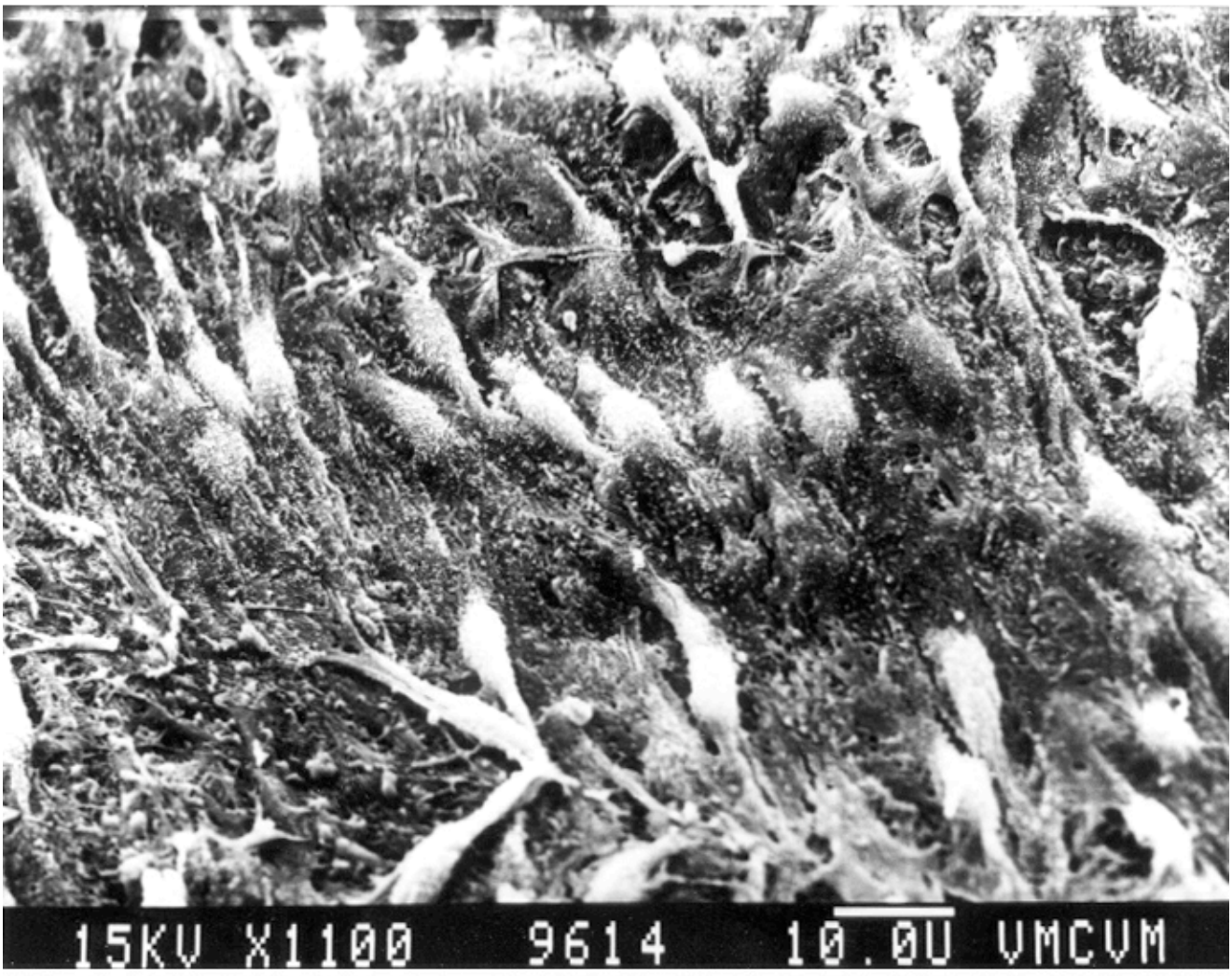


Figure # 1.9: Experimental day #4

Figure # 1.10: This micrograph, antemortem day 7 post injection (10^5 CFU PH, IP), shows an increase in the fibrillar components on the endothelial surface as compared to previous days micrographs.. There appear to be ulcerations of the endothelium at 11 and 12 o'clock. Spherical cell-like elements can be seen throughout the micrograph, consistant with erythrocytes fibrocytes and leukocytes. Overall there appears to be less organization to the direction and thickness of the fibrils noted previously (figures # 1.8 and # 1.9). The topography of the endothelial surface has been obscured by the fibrillar elements.

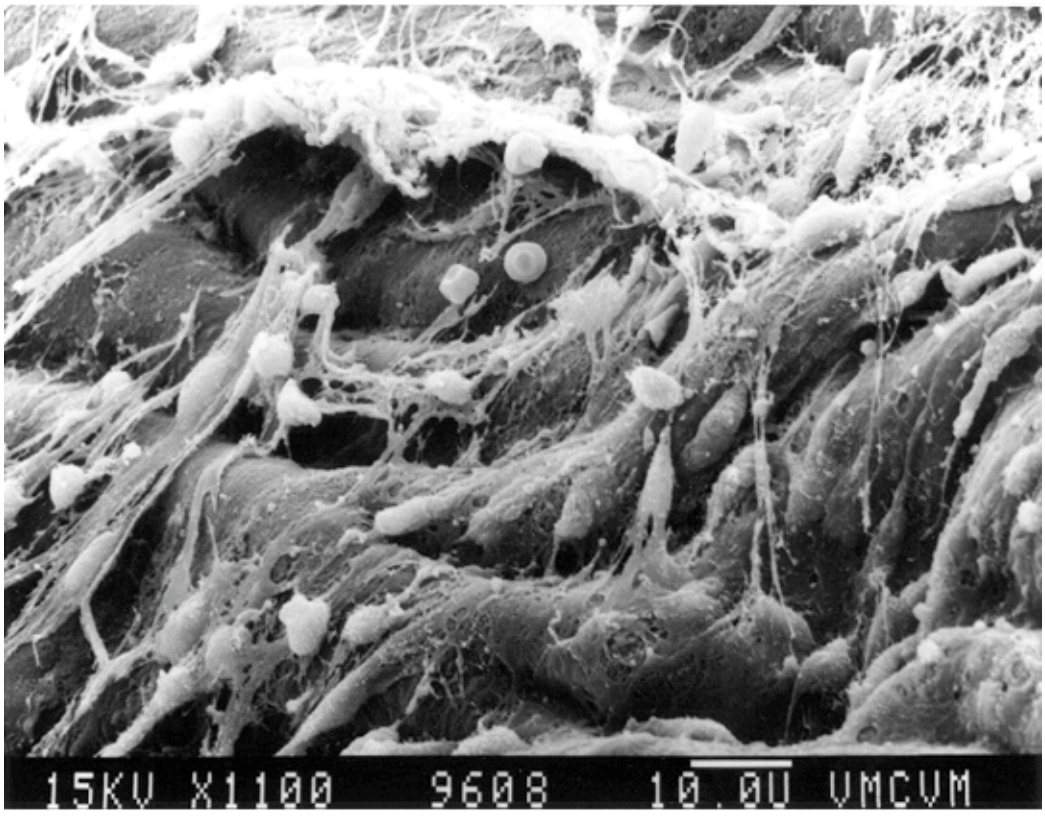


Figure # 1.10: Experimental day #5

Figure # 1.11: This micrograph has thicker (3-4 micron) fibrils in a network that is parallel to the rugae (endothelial folds) of the endothelial surface. The thicker fibrils appear to be organized multiple strands of the smaller (1 micron) fibrillar elements. There are two areas of bullae / vesicular rupture of the endothelial surface that can be seen at approximately 1 and 3 o'clock. Overall the topography of the surface is smoother than that of the negative control suggesting endothelial cell swelling.

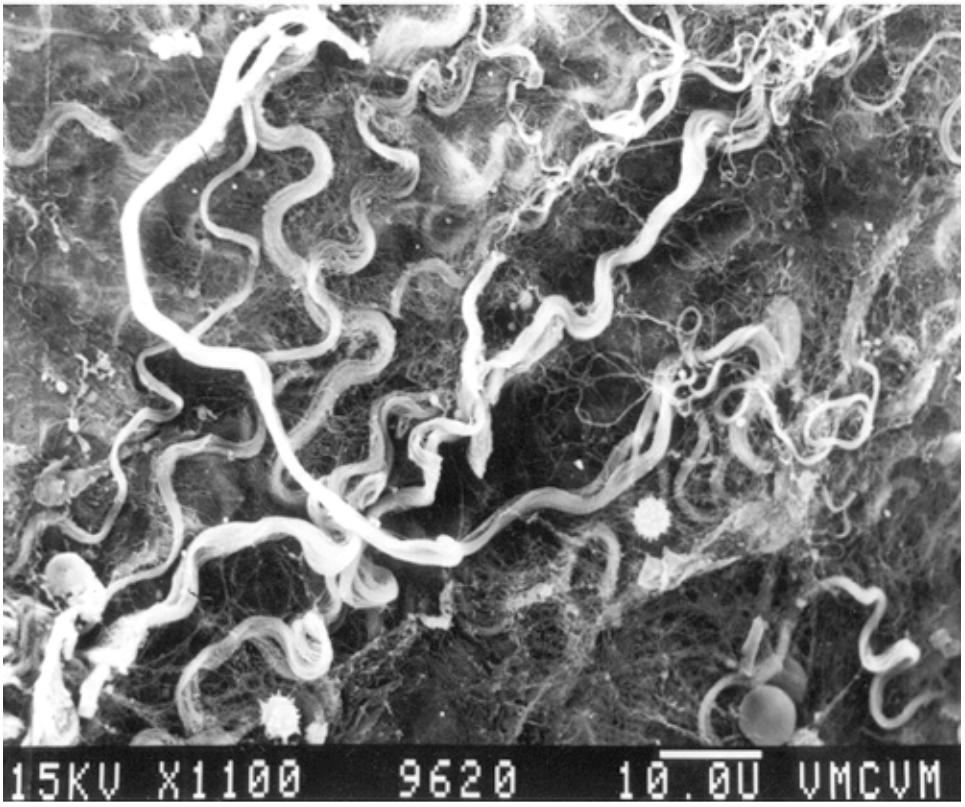


Figure # 1.11: Experimental day #6

Figure # 1.12: This micrograph day 7 post injection (IP 10^5 CFU-PH) demonstrates the maximum level of endothelial damage noted over all days examined (day 1 through day 21). There are multiple ruptured bullae, ulcerative areas exposing tissue consistant with a basement membrane. The luminal surface of the vascular endothelium appears to be devoid of rugae (endothelial folds) suggesting endothelial swelling. There is a minimal amount of fibrillar material scattered over the surface of the endothelium.

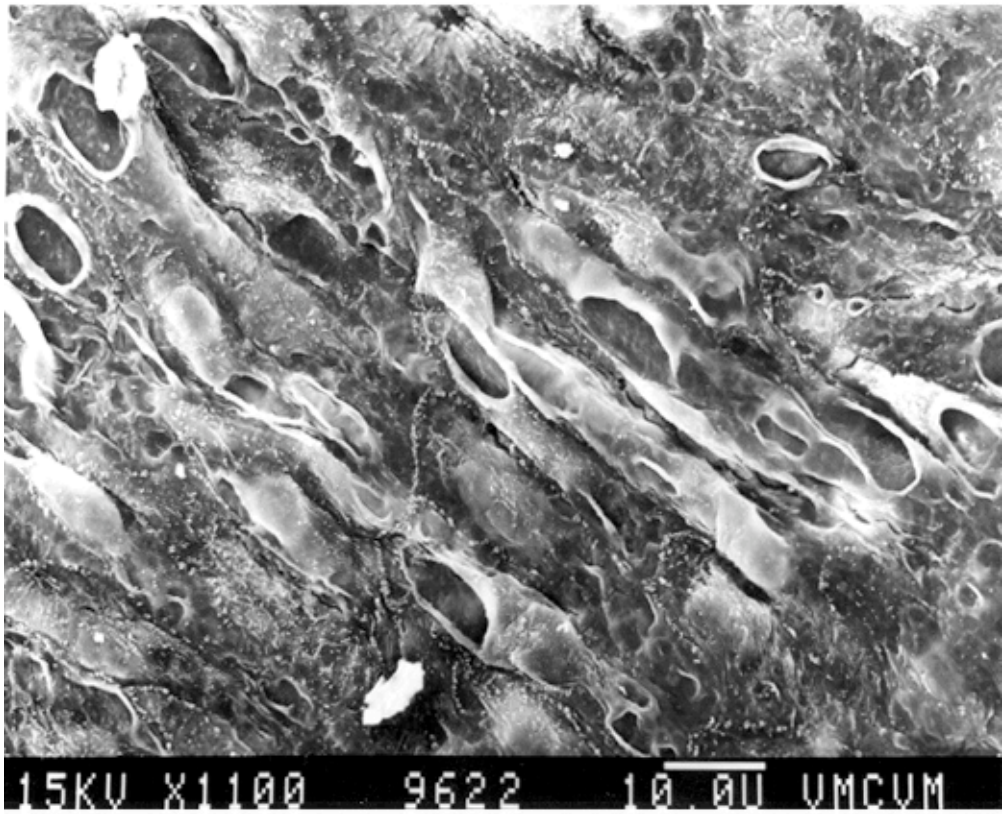


Figure # 1.12: Experimental day #7

Figure # 1.13: Day 14 demonstrates some resolving ulcerative damage at the 3 o'clock position. There are cellular elements in the lower left quadrant that are of a shape and size consistant with leukocytes. The endothelium is also devoid of the natural rugae (endothelial folds) noted in both the negative controls and earlier micrographs suggesting endothelial swelling. There are thin fibrillar strands consistant with fibrin. There are also fine longitudinal fissures that are likely due to fixation (dehydration) artifacts.

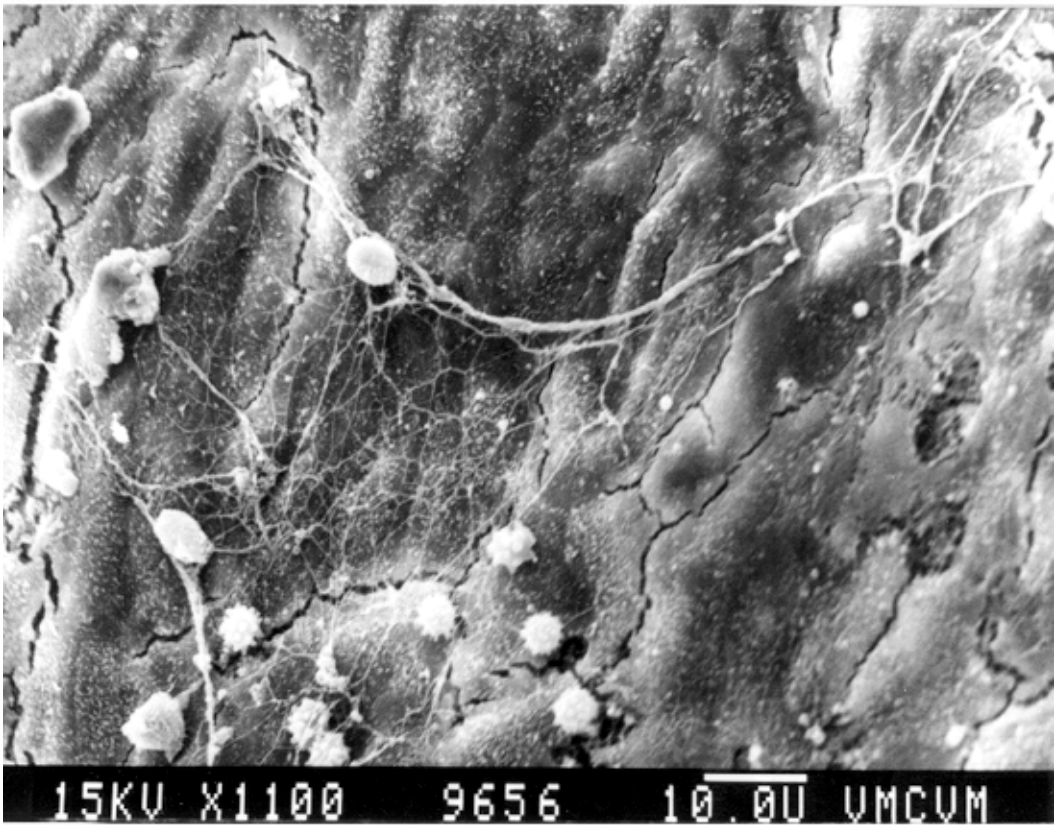


FIgure # 1.13: Experimental day #14

Figure # 1.14: Day 21 demonstrates moderate resolution of changes noted on prior experimental days. There are decreased cellular and fibrillar elements. The rugae (endothelial folds) appear to be increasing in size, consistant with resolving lesions, decreased swelling and an improving topography as compared to days 4 to 14 and the negative control. There are cellular elements (consistant with RBC). Noted at 9 and 11 o'clock there appear to be darkened circular areas that are consistant with resolving ulcerations / bullae. There are also fine longitudinal fissures consistant with fixation or preparation artifacts.

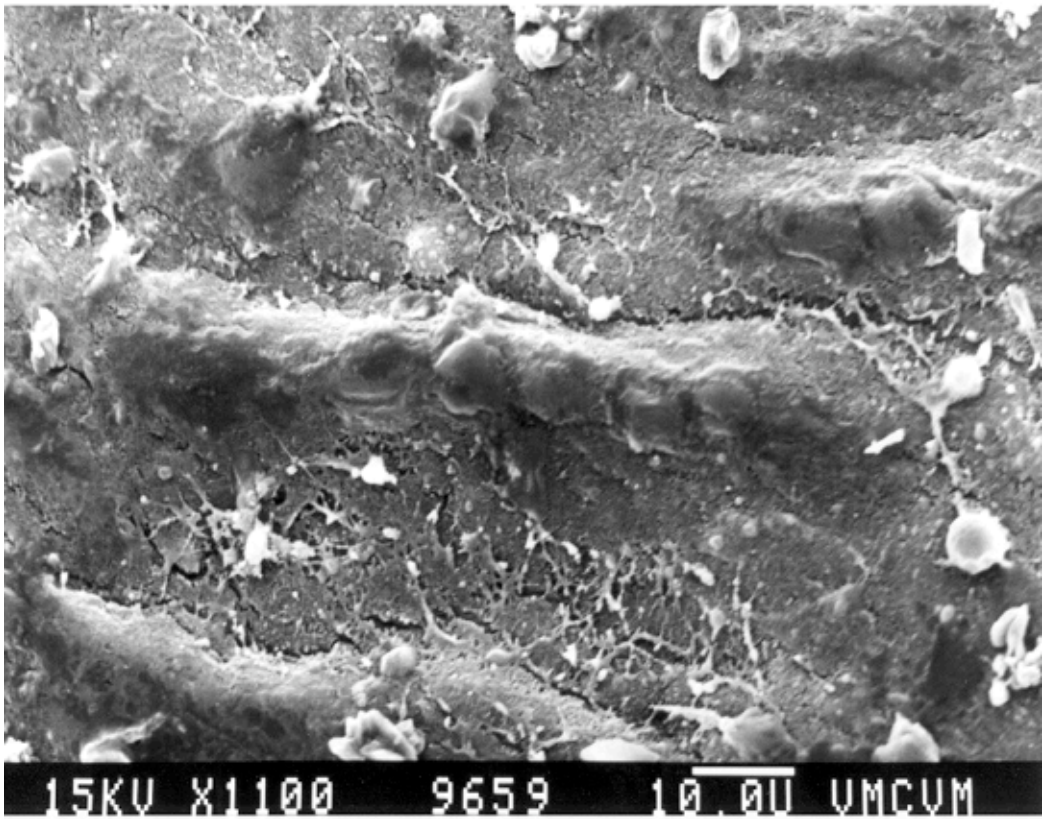


Figure # 1.14: Experimental day #21

Figures # 1.15 – 1.20: Scanning electron micrographs (ScEM) of the luminal surface of the aorta of the experimental Sprague-Dawley rat. Tissue sections represented are adjacent to those obtained for biophysical evaluation. Figure # 1.15 is the negative control (saline) , figure #1.16 is the positive control (10^5 CFU-PH) on day 3 post-injection. Figures # 1.17 through # 1.20 represent the experimental treatments as noted.

Figure # 1.15: This is a micrograph of the luminal surface of the proximal aorta following antemortem IP injection of sterile saline (day 3). There are minor visible discontinuities of the luminal surface, fibrillar strands and cellular elements (consistant with fibrocytes) homogeneously cover the surface. The endothelium topography is evenly colored (gray) throughout the micrograph. There are no signs of endothelial disruption, thrombocyte or leukocyte invasion.

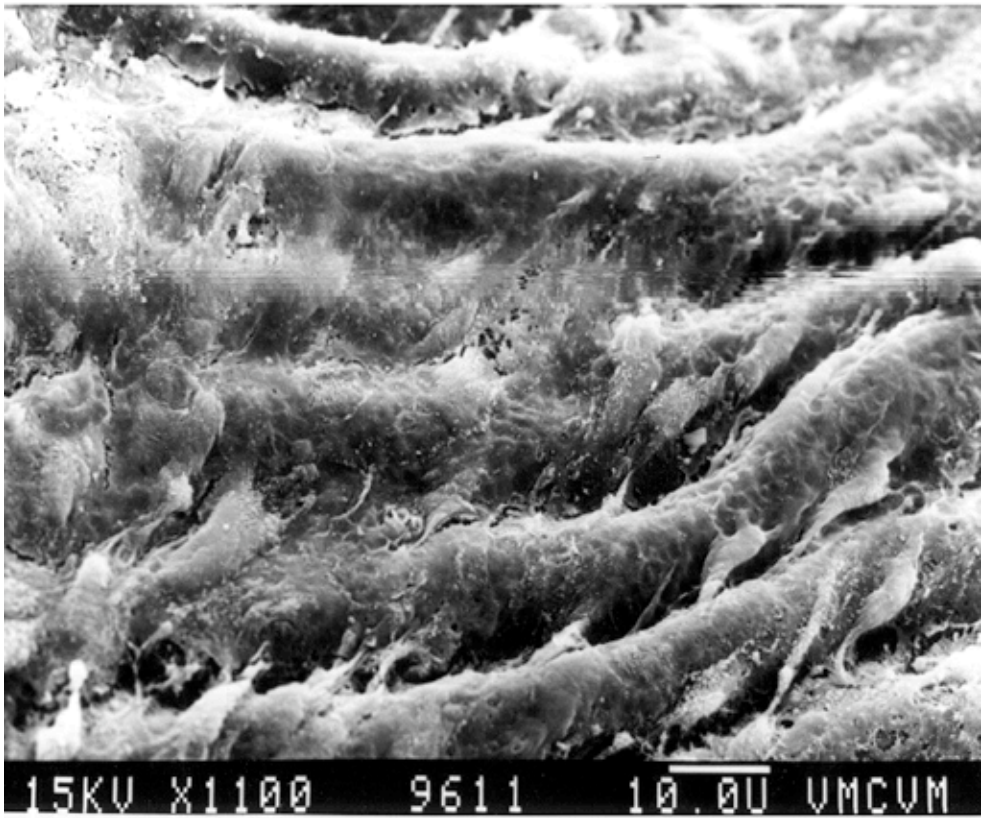


Figure # 1.15: Negative control (0 CFU PH, @ 3 days post-injection)

Figure # 1.16: This is a micrograph of the luminal surface of the proximal aorta following antemortem IP (3 day) injection of 10^5 CFU PH. There are significant disruptions of the endothelial surface at 11 and 12 o'clock positions. There are circular / spherical cell-like adhesions consistant in size and shape to thrombocytes at 1-2 and 7-8 o'clock positions. Other cell like adhesion are seen at 6-9 and 3 o'clock positions, these are consistant with leukocytes. Flattened cell like adhesions with fibrillar-like attachments and are consistent with fibrocytes. The endothelial topography is not as smooth as the negative control. There are increased depth and size of rugae (endothelial folds).

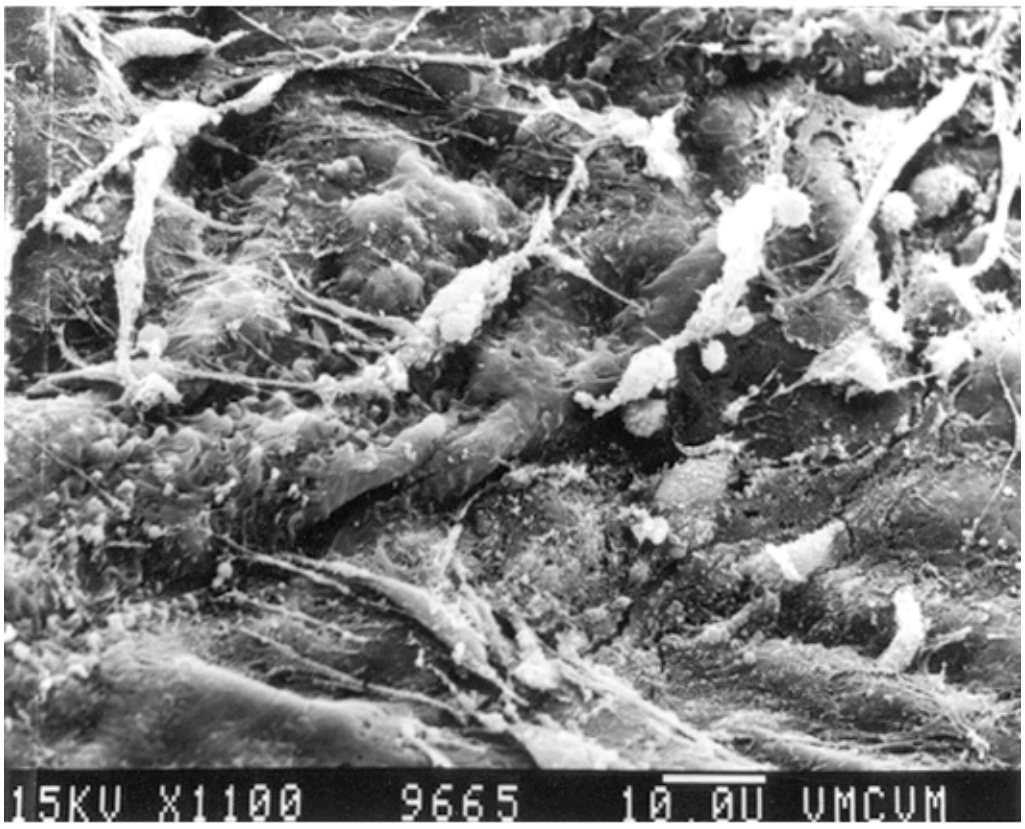


Figure # 1.16: Positive control (10^5 CFU PH, @ 3 days post-injection)

Figure # 1.17: This micrograph is of the luminal surface of the aorta after injection (10^1 CFU-PH IP) day 3. There is an increase in the cellular elements (consistant with erythrocytes and leukocytes) at the 7 o'clock positions. Fibrillar elements do not appear to have increased over the negative control. Overall the topography appears smoother, decreased rugae (endothelial fold) size suggesting swelling. There are shallow endothelial depressions at 7- 8 o'clock consistant with early bullae / vesicles.

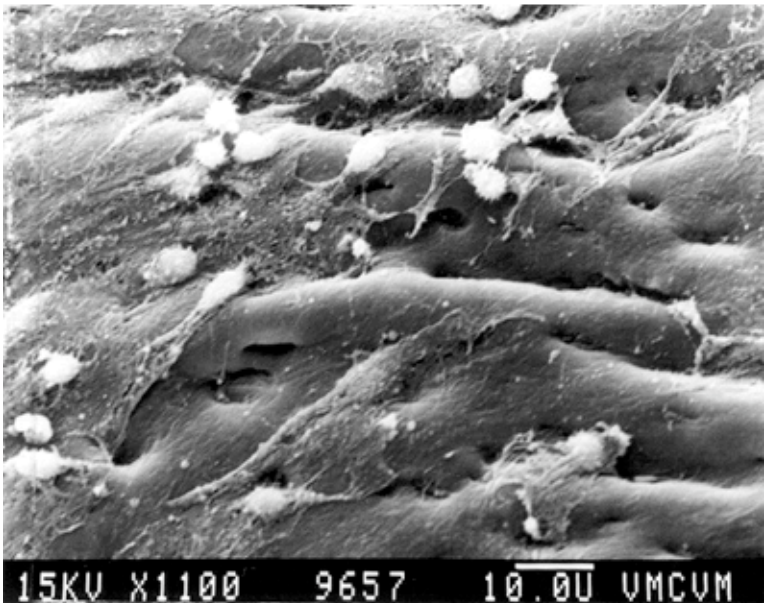


Figure # 1.17: Experimental dose 10^1 CFU of PH.

Figure # 1.18: This micrograph is of the luminal surface of the aorta after injecetion (10^1 CFU-PH, IP) day 3. There is an increase in the celular elements (consistant with erythrocytes and leukocytes) at the 7 o'clock positions. Fibrillar elements do not appear to have increased over the negative control. Overall the topography appears smoother, decreased rugae size (endothelial folds) suggesting endothelial swelling. There are shallow endothelial depressions at 7-8 o'clock position, consistant with early bullae / vesicles.

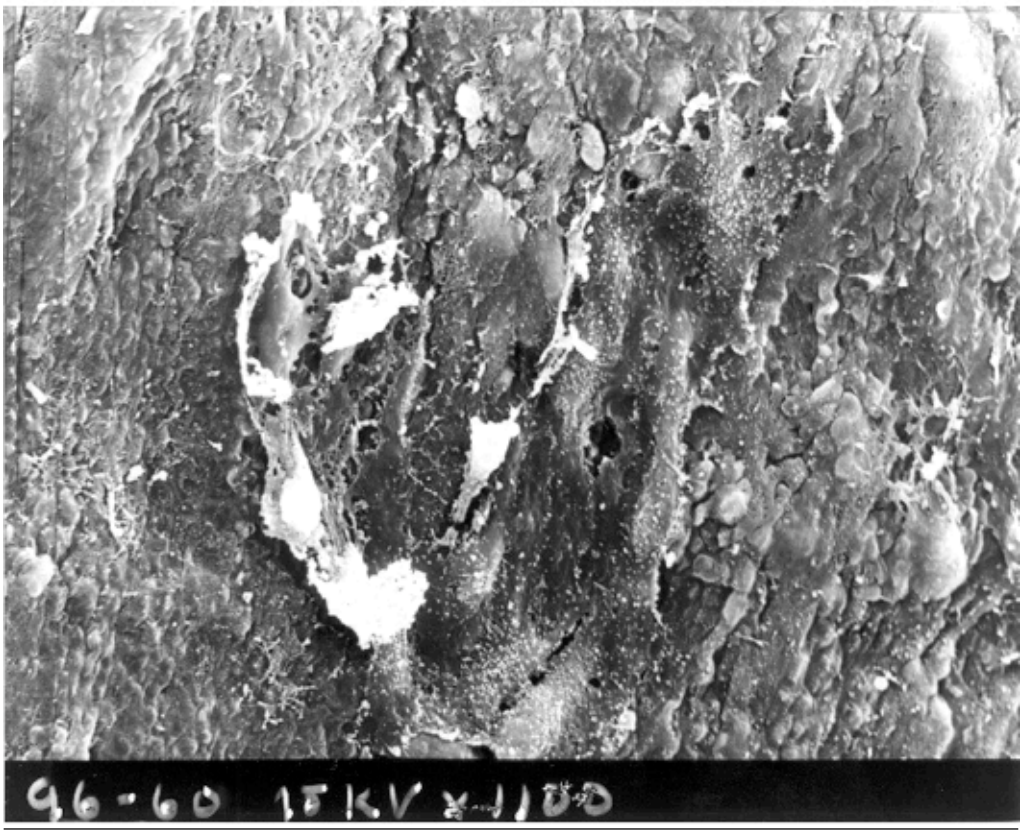


Figure # 1.18: Experimental dose 10^2 CFU PH

Figure # 1.19: This micrograph (10^3 CFU-PH IP) demonstrates evidence of increasing luminal / cellular activity. There are multiple disc-like cells with an apparent concave surface (consistant with erythrocytes) and spherical cells with small "projection" (consistant with leukocytes) The endothelium also appears to have a flattened topography. There are some irregular circular endothelial depressions or ulcerations at the 4, 7 and 12 o'clock positions. Overall there appears to be an increased level of cellular activity and endothelial swelling.

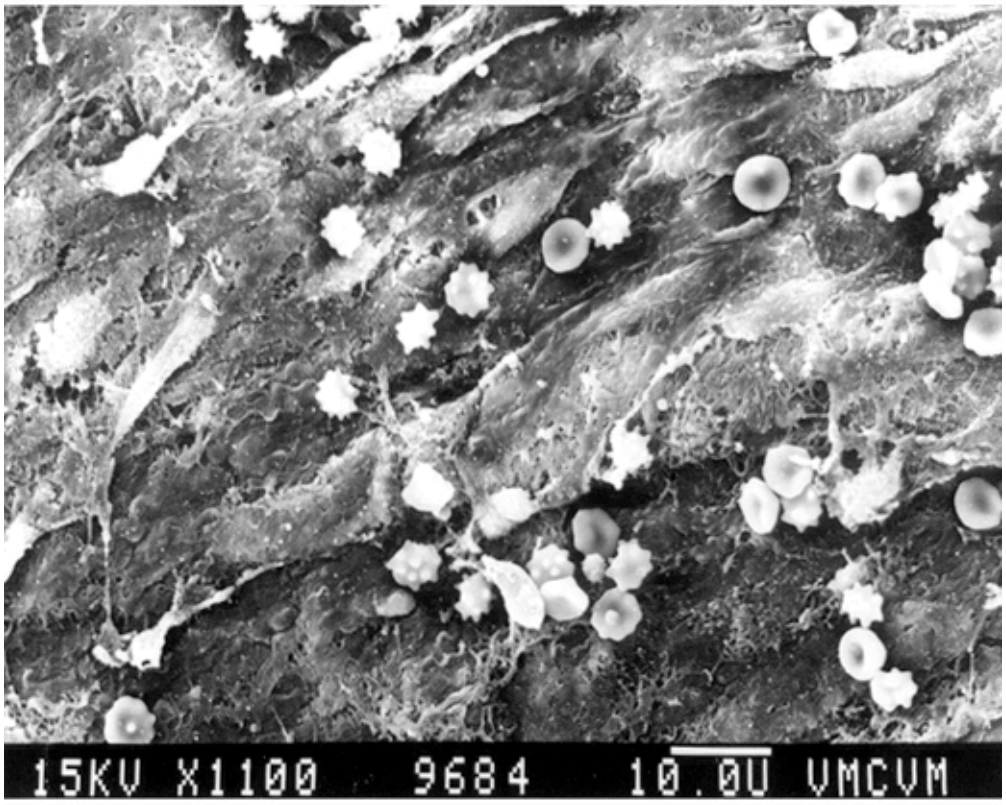


Figure # 1.19: Experimental dose 10^3 CFU PH

Figure # 1.20: This micrograph (10^4 CFU-PH IP) appears to demonstrate an increase in fibrillar activity over the endothelial surface. The cellular elements are consistant with leukocytes and fibrocytes. There are fibrillar elements consistant with fibrin. Few endothelial disruptions can be identified due to the excessive nature of the fibrin-like material over the luminal surface.

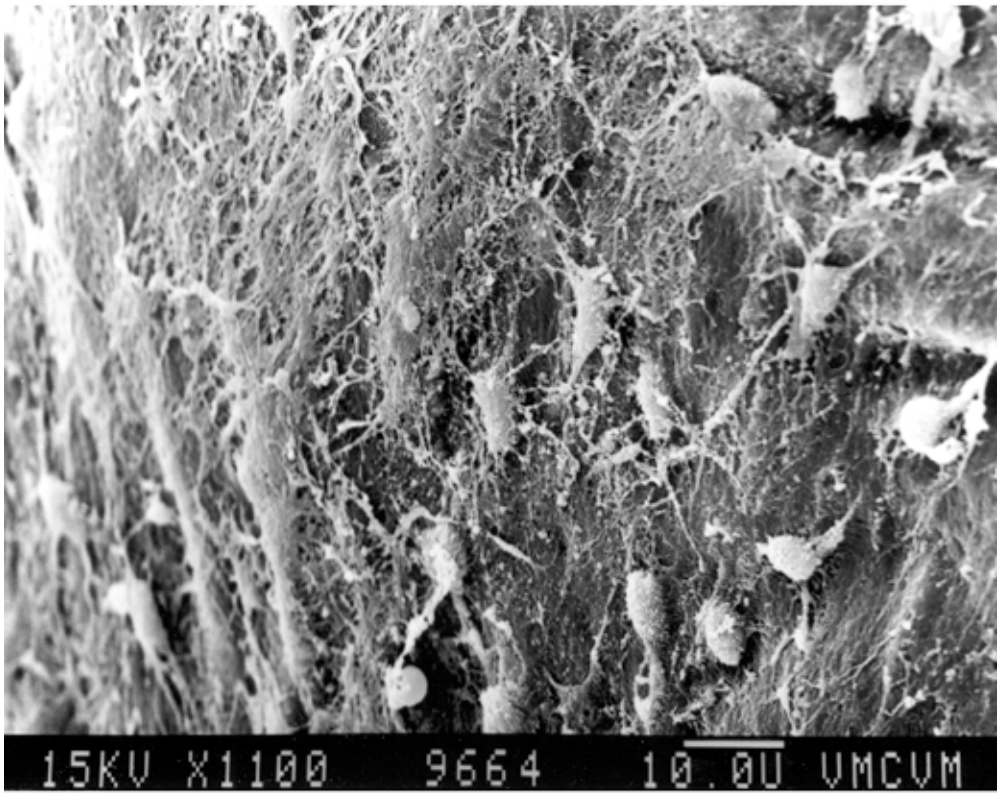


Figure # 1.20: Experimental dose 10^4 CFU PH

CONCLUSIONS

It is unclear as to whether the biophysical effects associated with isoproterenol occur solely as a result of the disruption of the beta adrenergic system alone or are a combination of the disruption of the beta adrenergic receptor and alpha-2 and possibly even alpha-1 adrenergic receptor dysfunction. The data indicating abrupt change from relaxation to an increase in tension (reversal of the trend), may be due to some interaction of isoproterenol at the alpha adrenergic receptor at higher concentrations. Isoproterenol is a beta adrenergic selective, not specific, drug. However, at higher concentrations the drug becomes less selective and has alpha receptor activity possibly confounding the drug selectivity (e.g. above 10^{-4} molar). This suggests that either the alpha-1 or alpha-2 receptors are responding to the beta adrenergic agonist or that the beta receptor activity for the remaining population of active receptors has been maximized for relaxation and are demonstrating a pharmacological failure. It is very difficult to isolate a single receptor type of the vascular system pharmacologically. In most cases, neuropharmacological activities are dynamic and interaction among receptor types is a common confound in these experiments.

It is apparent from the data that the maximal pharmacological effect of antemortem intraperitoneal injection of PH occurs at two days (48 hours) after exposure to 10^2 CFUs in the rat. The maximal endothelial, cellular and fibrillar (micro-anatomical) events, at the luminal surface, consistent with endothelial disruption, increase from day 2 or 3 through day 21 and appear to be at a maximum on day 7 (figure #12) or after 10^3 CFU PH-IP. The exact duration of the pharmacological / micro-anatomical disruption was not able to be determined within the limits of the present experiment. It is most probable that the duration of the beta adrenergic receptor system is in proportion to the quantity and duration of exposure to PH and LPS. It is likely that some component of an individual's immunological status (e.g. neutrophil population, cortisol concentration, level of cytokine release) are factors in the duration and extent of the disruption of the adrenergic mechanisms. It must first be ascertained whether the physiological effects noted, are a

result of pure beta adrenergic and endothelial disruption or if there is a component of alpha-1 or alpha-2 adrenergic system failure. It is less likely that the disruption of the vascular adrenergic dynamic control mechanisms are a consequence of only one receptor mechanism. The vascular smooth muscle homeostatic control system is modulated by the adrenergic, cholinergic and the non-adrenergic non-cholinergic (NANC) system. It is therefore very likely that multiple system changes are involved in the disruption of the beta adrenergic receptor mediated smooth muscle relaxation response.

The morphological results are consistent with earlier finding (Weekley 1993E). The changes noted are consistent temporally, with the disruption of beta adrenergic receptor mechanisms and the initiation of micropathology. This observation is consistent with inflammation and is likely to involve the endothelium, smooth muscle and immunologically active cellular elements.

These results support the hypothesis that disruption of beta adrenergic receptors is mediated by either PH or an element intimately associated with PH (e.g. LPS). The observation that the beta adrenergic receptor mechanism is disrupted for up to 21 days at levels of PH parenteral exposure of 10^1 CFU suggest that some whole unit vaccines have the potential to induce changes that my be deleterious to an animal's health. The consequences of this study are that the disruption of the beta receptor mechanism associated with the vascular smooth muscle is likely to have occurred but is unlikely to be the sole mechanism involved in the pathogenesis of either post vaccination illness or the pathogenesis of pneumonic pasteurellosis. There is indirect evidence to suggest that beta receptor systems, beyond those of the vascular smooth muscle receptor mechanisms are affected. Potentially the most devastating consequence of BARC disruption is the failure of the limiting of leukocyte, and more specifically neutrophil, oxidative enzyme and elastase release. Further, morphological evidence suggests that there are deleterious thrombogenic events that occur at the surface of the endothelium and increase in severity up to day 7 and then continually improves until day 21. Similar findings are noted for increasing PH antemortem injections.

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B). EVALUATION OF ESCHERICHIA COLI (O 55/B5) ENDOTOXIN IN INDUCING BETA ADRENERGIC RECEPTOR MECHANISM MEDIATED DYSFUNCTION OF VASCULAR SMOOTH MUSCLE OF EX-VIVO RAT AORTA

Live *Pasteurella haemolytica* (PH) injected intraperitoneally in the rat, has been demonstrated to cause a disruption of the vascular beta adrenergic system associated with smooth muscle (Weekley 1993A; 1993B; 1993E). In the intact pulmonary vascular system the beta adrenergic receptor acts to mediate vascular dilation. On the arterial side this vasodilation increases the functional blood flow to the lung. The post-arterial beta adrenergic receptor mechanism also causes vasodilation where the consequence is increased drainage of blood from the lung which prevents pulmonary hypertension and subsequent pulmonary congestion. Therefore any disruption of venous dilation has the potential to cause either pulmonary vascular congestion or pulmonary interstial edema, two of the many classical signs of pneumonic pasteurellosis.

Many authors have examined the epidemiology of the efficacy of several monovalent PH vaccines. The results of these studies suggest that vaccination is less than efficacious in preventing disease in the short term and may even predispose the animal to an increased risk of disease (Martin 1983; Thorlarkson 1990). It has been demonstrated that parenteral exposure to a live vaccine, Precon® (live vaccine), and an endotoxin free vaccine, Presponse® (leukotoxoid rich), causes disruption of beta adrenergic receptors associated with vascular smooth muscle in several species (Weekley 1991A; 1993B). Beta receptor mechanisms are associated not only with the vascular smooth muscle, but also have been demonstrated to ameliorate extravasation of fluid, inhibit thrombin interactions at the endothelial surface and mediate excessive release of destructive enzymes and oxygen free radicals from leukocytes, particularly neutrophils. The importance of the beta receptor system to pulmonary homeostasis is well established. The beta adrenergic receptor mechanism disruption may be significant factor in the post-vaccination increase in disease morbidity and mortality, the pathogenesis of pneumonic pasteurellosis and the severity of pulmonary vascular lesion development.

The question of which component of the monovalent pneumonic pasteurellosis vaccines being examined is causing the beta adrenergic dysfunction has yet to be investigated. Each manufactured vaccine is unique both in its immunomodulatory elements and in the chemical adjuvants used to enhance the host immunoprotective reaction. Four virulence factors have been described for PH A-1 (PH). These include: fimbriae, polysaccharide capsule, leukotoxin, and lipopolysaccharide (endotoxin, LPS). The most likely portion of the vaccine to cause beta adrenergic complex failure is endotoxin. However scientific information concerning Presponse® indicated that this vaccine was endotoxin free as assessed by the Limulus Ameobocyte Lysate assay (LAL) (Cyanamid 1992). Researchers have suggested that Presponse® is not endotoxin free but may, coincidentally, contain a factor that causes a false negative test in the LAL assay (Maheswaran et al 1995). Endotoxin has been demonstrated to disrupt the beta adrenergic receptor system of cardiac beta adrenergic mechanisms (van Heuven-Nolsen 1986; Campbell 1993). The evaluation of endotoxin as a factor in the disruption of the vascular smooth muscle associated beta adrenergic receptor mechanisms now appears to be an important question to investigate.

Endotoxins have four common elements among the gram negative bacteria that share this feature. All endotoxins contain four basic components, lipid A moiety, the inner core, the outer core and the o-specific polysaccharide chain. The species differences in endotoxin are found in the sequences associated with the o-specific-polysaccharide side chain. The lipid A moiety of endotoxin is likely the essential factor causing the defect noted by Weekley. Due to difficulties in isolating and purifying PH endotoxin, *Eschericia coli* (EC) endotoxin has been selected for use. EC endotoxin has been shown to uncouple beta adrenergic receptors of the myocardium (Romano 1985; Shepard 1987; Forse 1989; Campbell 1993; Bensard 1994). The extraction and purification of *EC* endotoxin has been standardized and well characterized and is available commercially. Two studies have directly compared the effects of EC endotoxin to that of PH and found that the EC endotoxin was less virulent than the PH endotoxin (Rimsay 1981; Lacroix 1993). Unfortunately,

neither of these studies investigated the functioning of the vascular beta receptors with respect to the activity. It is for these reasons that EC endotoxin was chosen for this study. This paper proposes to examine the effect of purified EC endotoxin alone as one factor affecting the functioning of vascular beta adrenergic receptors associated with smooth muscle in the rat aorta previously described by Weekley (1991A; 1993A; 1993B).

MATERIALS AND METHODS

A) Treatments and Subjects

Adult male Sprague-Dawley rats (Harlan Sprague-Dawley Inc., Indianapolis, Indiana) 150-175g were used in this series of experiments. The animals initially were housed in a quarantine area for a 1 week for acclimatization to the new facility and observe for physical signs of disease. The acclimatization period involved a 12:12 light dark photo period, room temperature was 22^0 C +/- 3^0 C. Each experimental animal was injected, intraperitoneally, with 200 micro endotoxin units (μEU) of EC endotoxin in sterile, endotoxin free saline (O55:B5, Sigma L2880). Negative control animals were injected with endotoxin-free normal sterile saline, in the same manner as the experimental animals. The positive controls were injected with 10^5 colony forming units (CFU) of PH intraperitoneally as described by Weekley (1993E). There were four animals in each of the three groups (n=4)

B) Preparation of Drugs and Solutions

The PH suspension was evaluated for its endotoxin content by use of the Limulus Ameobocyte Lysate assay (QCL-1000, Bio-Whittaker Co.). A standard preparation of 10^5 colony forming units was evaluated at several concentrations by log-serial dilutions. Standardized equivalent EU *Escherichia coli* endotoxin (Sigma L2880, O55:B5) was added to sterile endotoxin

free saline and vortexed in a sterile injection vial to ensure complete mixing. The appropriate quantity (200 μEU) of EC endotoxin was then immediately injected, intraperitoneally in the experimental animals.

Tissues were carefully dissected and suspended in Krebs-Henseleit saline buffer. This buffer was composed of one liter of sterile double distilled water in a sterile 5 liter Erlenmeyer flask plus: 6.9 grams NaCl ; 0.35 grams KCl; 0.28 CaCl; 0.14 grams $MgSO_4$; 2.09 grams $NaHCO_3$; 0.16 grams KH_2PO_4; 2.0 grams dextrose, as described by Weekley (1994). This solution was aseptically mixed immediately before use.

Potassium chloride was used for initial smooth muscle contraction. This solution was prepared in sterile, double distilled water (30 millimolar concentration when 200 microliters of stock KCl solution is added to 30 milliliters of the bathing solution).

Isoproterenol, a mixed beta adrenergic receptor agonist, was used to evaluate the beta receptor functioning of isolated, ex-vivo, aortic vascular tissue. This solution was prepared in sterile, double distilled water. Serial decreasing logarithmic concentrations of isoproterenol were made from the initial 10^{-2} molar solution. The serial dilutions were made by taking 100 microliters of the initial stock solution and diluting this in 900 microliters of sterile double distilled water sequentially to 10^{-9} molar solution.

C) Tissue Collection and Measurement Techniques

Subjects were anesthetized to a surgical plane of anesthesia with sodium pentobarbital (80 mg/kg, IP) which was then followed immediately by a second injection of 25 IU of sodium heparin (IP). When at a deep surgical plane of anesthesia, the abdomen was opened to reveal the diaphragm. The approach to the thorax and thoracic aorta was through the diaphragm. The

ventrum of the thorax was removed to completely expose the thoracic contents. The aorta was removed from the base of the heart to the last rib ensuring that minimal damage or stretching occurred. The entire vessel was the placed in warm aerated Krebs-Henseleit (37^0 +/-2^oC, mixed with 95% oxygen: 5% carbon dioxide) for further dissection and complete removal of extraneous fat and superficial tissue. All aortic rings were obtained from the base of the heart. The rings were carefully measured to be 3 mm in width. The dissection process was performed with exceptional care to ensure that stretching or damage to the endothelium was avoided.

The 3 mm aortic ring, removed for biophysical testing, was placed between two glass hooks in a thirty-five milliliter tissue bath. Changes in tension (isometric contraction) of the aortic ring were measured following the addition of known concentrations of various drugs. One hook was attached to a fixed point at the bottom of the tissue chamber while the other hook was attached to an electronic transducer that was calibrated to give output in grams tension. The tissue was placed in an insulated heated tissue bath containing Krebs-Henseleit bicarbonate buffer The buffer was aerated with 95% oxygen and 5% carbon dioxide through each of four individually isolated tissue baths. A constant tissue bath temperature of 37 ^{O}+/- 2^o C was maintained by circulating water through a temperature controlled water pump and sequentially through the outer heating jacket of each tissue bath. Each tissue ring initially was placed in the tissue bath under 2 grams resting tension for two hours and allowed to equilibrate. The Krebs-Henseleit buffer was replaced every 15-20 minutes throughout equilibration and testing. Testing started immediately after the equilibration period. The aortic rings, in the tissue bath, were exposed to 200 μl of potassium chloride (KCl, 30 millimolar) and allowed to equilibrate to this treatment over 30 minutes. After the tissue had equilibrated to the KCl, the recording was started and sequential increasing molar doses of isoproterenol were added. Isoproterenol hydrochloride was used to evaluate the response of beta adrenergic receptors in causing smooth muscle relaxation.

Electronically calibrated analog signals were transmitted from the transducer connected to the glass hook through an analog digital converter (MacLab A/D signal converter, World Precision Instruments). Digitized information was captured by MacLab data acquisition software (MacLab 2.51) on a Model M5011 Macintosh SE computer (1MB RAM, 800 K drive, 20 megabyte hard disk) in real time. Records of vascular smooth muscle activity, as a cumulative dose response to log molar increases in isoproterenol, were obtained and the results stored on a 3.5 inch computer disk.

D) Statistical Analysis of Data

Data were analyzed in several ways. Initially the data were compared for each experimental treatment at the isoproterenol concentration of 10^{-5} molar. In pilot studies this concentration of isoproterenol appeared to approximate the effective concentration 50% (EC_{50}) and so was selected to be an appropriate point for comparison of different treatments for the experimental protocols. The comparison was completed by use of an analysis of variance (ANOVA) using group (n=4) means and standard error of the mean. This comparison was to evaluate significant differences among treatment groups.

Further analysis consisted of a pair wise comparison of the values of 0 molar isoproterenol and 10^{-5} molar isoproterenol. Pair wise comparisons were accomplished by use of Student-Newman-Keuls test. This comparison was to evaluate change, with respect to relaxation or contraction, with increasing logarithmic doses of isoproterenol

RESULTS

Comparison of the effects of *Escherichia coli* endotoxin (EC, 055:B5) with the whole organism preparation of PH (10^5 CFU) suggests that the EC endotoxin mediates similar beta

receptor deficits as was noted to be caused by IP injection of a suspension of the whole PH organism (figure #1). The mechanisms and pathogenesis of this deficit remains to be investigated.

The negative control resulted in the expected smooth muscle relaxation in response to increasing logarithmic molar isoproterenol. The positive control failed to respond to the isoproterenol cumulative dose response curve, as expected. Statistical comparison of the three groups at 10^{-5} molar concentration of isoproterenol revealed that there is a highly significant difference between the negative control and the positive control ($p < 0.001$). The aortic rings from the experimental animals treated with the EC endotoxin were identical to the positive control animals treated with PH endotoxin at 10^{-5} molar isoproterenol (no significant difference, $p > 0.05$). At concentrations of isoproterenol above 10^{-5} molar, the aortic rings from the EC endotoxin treated animals show a greater increase in tension than the positive controls. The results were not statistically different at either 10^{-4} and 10^{-3} molar isoproterenol ($p > 0.05$).

DISCUSSION

The results of this experiment suggest that EC endotoxin alone was sufficient to cause disruption of the beta adrenergic receptor mediated vascular smooth muscle function. This evidence supports Maheswaran that Presponse® likely contains endotoxin though the significance of this finding has yet to be evaluated as to its affect on the in-vivo pulmonary vascular BARC (Maheswaran 1995) Further, the results of this experiment are in agreement with current literature indicating that EC endotoxin disrupts cardiac associated beta adrenergic receptor mechanisms (Romano 1985; Shepard 1987; Forse 1989; Campbell 1993; Bensard 1994).

There are differences between the EC endotoxin and that of the PH endotoxin. The most striking of these differences is that EC endotoxin has O-specific polysaccharide side chains not present on the PH endotoxin. Several authors have suggested that the lipid A moiety of the

endotoxin is responsible for the deleterious activity of LPS in susceptible hosts. Since the lipid A moiety is relatively conserved in all bacterial LPS, the results presented here should be as valid for PH endotoxin as for EC endotoxin. Comparative studies suggest that EC endotoxin is less destructive than PH endotoxin (Rimsay 1981; Lacroix 1993). One might expect that the EC endotoxin would cause less of a receptor interference than is experimentally observed with the PH control. The lack of difference between the receptor inhibition of EC LPS and PH A1 may be explained in several ways. It is possible that a separate factor associated within the PH organism restricts the full virulence of the lipopolysaccharide responsible for the beta receptor disruptive activity. Another explanation may be that total inhibition of the smooth muscle response occurs so that at any level of endotoxin at three days post exposure ther is a single, maximal response (ceiling phenomenon). Any increase beyond the minimally necessary level of endotoxin to cause system disruption fails to result in an increase in the dependent variable (beta adrenergic receptor mechanism dysfunction). Finally, the endotoxin level of sequentially prepared PH aliquots may vary to some degree. In assessing the concentration of PH at 10^5 CFUs one is only evaluating those organisms that are viable and result in a colony.

Endotoxin is an integral part of the gram negative wall of the organism. The fact that an organism is no longer viable doesn't reduce the endotoxin concentration. This fact makes it difficult to exactly reproduce the concentrations of LPS from Weekley (1993A, 1993E). One can see however that the disruption of the vascular smooth muscle is similar in magnitude and extent for both EC endotoxin and PH endotoxin. Previous experiments with varying concentrations of PH organisms, revealed a beta adrenergic system disruptive effect at PH concentrations as low as 10 viable PH organisms injected intraperitoneally. Evaluation of the minimal concentrations of EC endotoxin necessary to induce beta adrenergic associated vascular smooth muscle dysfunction would be interesting.

Figure # 1: Comparison of 200uEU of E. coli endotoxin with the PH positive and negative controls

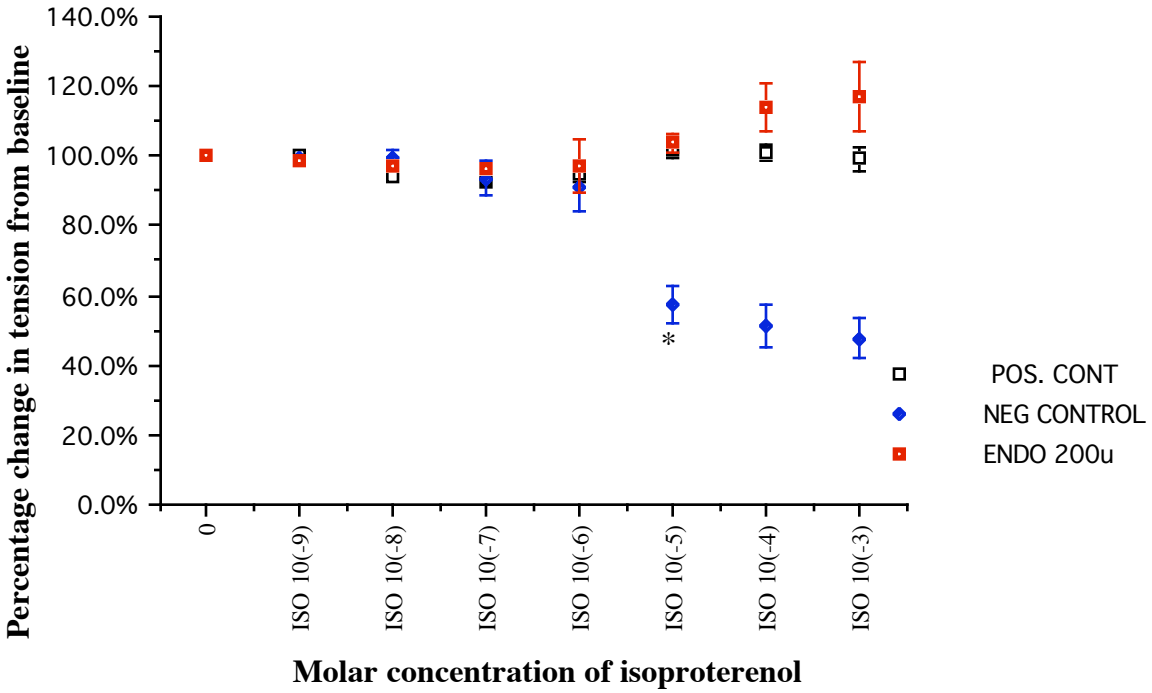

Figure # 2.1: The effect of*Escherichia coli* (ENDO 200u) endotoxin (Sigma L 2880, O55:B5) or live PH organisms (10^5 CFU, positive control) on beta adrenergic receptor mediated relaxation by isoproterenol and is reported as Means (+/- SEM). Subjects (n=4), were treated (IP injection of test substance) 3 days before tissue collection. There were no significant differences ($p > 0.05$) between the EC and PH treated subjects (*). There was a significant difference ($p < 0.001$) between the negative control and the two other experimental treatments.

There remains much work to do in this area. First, it remains to be shown that the endotoxin from PH is equivalently virulent as the EC endotoxin with respect to the vascular beta adrenergic receptors. There are four portions to the lipopolysaccharide molecule. It will become important to identify which of these components or combination of these components are active in the disruption of the beta adrenergic receptor. This information would allow the isolation and elimination of the offending moiety while retaining the portions of the microbiological organism that are important in immunostimulation aspects of the vaccine. Further experimentation using each of the four virulence factors recognized for PH, with respect to the beta adrenergic receptor system would result in additional useful information to the nature of the virulence mechanisms of the PH organism.

CONCLUSIONS

1) The evidence presented here gives a strong suggestion that EC endotoxin alone, administered parenterally is sufficient to cause vascular smooth muscle associated beta adrenergic receptor disruption similar to that seen with exposure to PH organisms.

2) The definitive experiment using isolated purified PH lipopolysaccharide has yet to be completed. The characterization of the PH endotoxin could be of significance in determining the mode of action and toxicokinetics with respect to the disruption of the BARC.

3) Similar experiments using the various isolated and purified components (e.g.the lipid A inner core, outer core or o-specific polysaccharide chain) of the endotoxin would also be enlightening.

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C). CHANGES IN BETA ADRENERGIC STIMULATED CYCLIC ADENOSINE MONOPHOPHATE LEVELS (cAMP), IN EX-VIVO AORTIC RINGS, FOLLOWING IN-VIVO, INTRA-PERITONEAL EXPOSURE TO *PASTEURELLA HAEMOLYTICA* A-1 IN RATS

The exact pathogenesis of pneumonic pasteurellosis is unclear. Weekley has demonstrated, in rats, that a single intraperitoneal injection of 10^5 CFU of *Pasteurella haemolytica* A-1 in rats can result in an elimination of the beta adrenergic mediated aortic smooth muscle relaxant response to isoproterenol (Weekley 1993A). Similar results have been described for the pulmonary vasculature of cattle (Weekley 1993B) and sheep (Weekley 1993E). Beta adrenergic receptors (BAR) are intimately involved with vascular homeostasis. Beyond the effects of the modification ot the release of oxidative enzymes elaborated by leukocytes, the beta adrenergic receptor may also act in several key ways to inhibit the release of potentially severely destructive endogenous elements. The significance of this to the establishment of pneumonic pasteurellosis in ruminants is not understood. Evidence exists to support a relationship between the disruption of the beta adrenergic receptor and development of the clinical and pathological signs of respiratory disease in cattle.

Beta adrenergic receptors associated with the vascular endothelium have a role in regulating the fluid dynamics between the intravascular and extravascular microenvironments (Mizus 1985; Minnear 1986). It is necessary to regulate the extravasation of vascular fluid, electrolytes and osmotic factors to maintain health. Other beta adrenoceptors modulate the elaboration of tissue factor, the first step of the extrinsic pathway of coagulation (Busso 1991). Tissue factor generation causes the release of thromboplastin and prothrombin which is then converted to thrombin and fibrinogen. The presence of fibrin attached to the luminal surface of the vessel is a characteristic of the endothelium noted to occur in response to a PH challenge in rats and cattle (Majno 1961; Weekley 1993A).

The beta adrenergic receptor is a critical factor in the control of leukocyte antibacterial responses both in-vitro and in-vivo (Bourne 1971; Ignarro 1974; Zurier 1974; Hills 1975; Harlan 1981; Busse 1984; Engels 1985; Tecoma 1986; Warren 1987; Barnard 1992; Kubes 1993; Granger 1994; Seccombe 1994; Al-Essa 1995). Increased pulmonary vascular trapping and margination of leukocytes coupled with an interruption in the beta adrenoceptor mediated inhibition of the neutrophil may lead to a catastrophic release of free radicals and proteases. This could result in pulmonary and vascular tissue destruction. It has been demonstrated that neutrophil deficient animals suffer less pulmonary tissue damage than neutrophil sufficient animals (Slocombe 1985; Breider 1991). Similar findings have been reported in the kidney (Paller 1989) and in the liver (Jaeschke 1990) of rats for various inflammatory disease states. Other evidence suggests that the beta receptor on macrophages can inhibit the release of pro-inflamatory cytokine products such as Tumor Necrosis Factor-alpha (Hu 1991; Severn 1992).

With respect to the bovine respiratory system, there are a number of unique anatomical features that make the vascular smooth muscle relaxation effects of BARs necessary to maintain optimum health. The bovine pulmonary vasculature has a thicker tunica muscularis compared to other mammals (Robinson 1984; Breeze 1985; Weekley 1995; Wren 1995). The pulmonary vasculature controls blood flow, at the capillary / acinus level, by manipulation of vascular constriction and dilation. The mechanisms involved in the dynamic control of the vasculature are varied and involve cholinergic, adrenergic, non-cholinergic non-adrenergic systems and humoral mediators (Breeze 1985; Barnes 1986; Barnes 1995). The adrenergic system is involved with the establishment of an optimal ventilation / perfusion match at the level of the pulmonary acinus (Green 1982; Barnes 1995). The beta adrenergic receptor is one factor in the metabolic equation that acts to mediate vascular relaxation and balance the vasoconstrictive effects of the alpha-1 and alpha-2 beta adrenergic receptors.

Disruption of beta adrenoceptor vasodilation may result in a shift of the dynamic vascular smooth muscle balance to result in an exaggerated alpha adrenergic mediated vasoconstriction. The consequences to the pulmonary and vascular tissue may include tissue hypoxia further enhancing vascular constrictive responses, metabolic acidosis, and increased trapping of circulating leukocytes, especially neutrophils in the capillaries and post-capillary venules.

The importance of the regulatory role of beta adrenoceptor on leukocytes, in the pulmonary vasculature smooth muscle and on the vascular endothelium, is well established (Engels 1985, Ogunbiyi 1986, Borregaard 1988, Bensard 1996, Opdahl 1993, Secomb 1994, Ignano 1974, Bourne 1971, Ignano 1974, Al Essa 1995). The devastating consequences of the uncoupling of this receptor is a logical explanation for some of the signs of the pathogenesis of pneumonic pasteurellosis. Evidence exists that suggests that the virulence factor, endotoxin, elaborated by Gram negative bacteria may be involved in the uncoupling of beta receptors (van Heuven-Nolsen 1986). Endotoxin acts directly on the beta adrenergic receptor to decrease receptor expression (van Heuven-Nolsen 1986; Forse 1989). There is a significant decrease in receptor density on cardiac myocytes in the presence of endotoxemia in human patients (Shepard 1987). Still other studies have demonstrated a diminished response of the beta-1 cardiac adrenoceptor distal to the agonist-receptor interaction (Campbell 1993). This additional evidence indicates that the beta adrenergic dysfunction, in the presence of endotoxin, may be responsible for the lack of cardiac response to beta-adrenergic agonists in endotoxemia (Romano 1985; Bensard 1994). One may expect the vascular system to be equally susceptible to endotoxin and beta adrenergic receptor dysfunction, This may explain some of the pathological and clinical signs of pneumonic pasteurellosis. Given these findings and the evidence presented by Weekley et al (1993E) it is important to explore the beta adrenergic receptor dysfunction of aortic tissue, exposed in-vivo to *Pasteurella haemolytica* A-1.

The beta adrenergic receptor is a G-protein linked receptor protein. Agonist ligand binding activates a G-protein intracellular linkage which in turn initiates the conversion of ATP to cAMP by the inducible enzyme adenylylate cyclase. cAMP then binds to regulatory subunits causing the liberation of active catalytic subunits that phosphorylate protein substrates. In vascular smooth muscle tissue, the result is that there is a relaxation and subsequent vasodilation of the affected vessel.

It is not clear why the vascular smooth muscle fails to respond to isoproterenol stimulation after PH administration. It is possible that the failure of the vascular smooth muscle to relax may be due to the disruption of the intracellular cascade of events associated with the beta adrenergic receptor. Numerous sites, within the post-receptor cascade, may be affected by slight biochemical, pH and biomolecular changes resulting in the disruption of the beta adrenergic receptor's ability to initiate vascular smooth muscle relaxation. Finally disruption of smooth muscle relaxation may exist within the vascular smooth muscle itself, either with respect to the signal / muscle interface (myosin light chain kinase) or within the smooth muscle proper (calcium flux and channel disruptions). To date, none of these possibilities have been examined. This paper proposes to examine the receptor and post-receptor cascade to establish if a defect exists between the receptor and the elaboration of the second messenger cAMP.

MATERIALS AND METHODS

A) Treatments and Subjects

Adult male Sprague-Dawley rats (Harlan Sprague-Dawley Inc., Indianapolis, Indiana) 150-175 g were used in this series of experiments. The animals initially were housed in a quarantine area for a 1 week for acclimatization to the new facility and observed for signs of

disease. The acclimatization period involved a 12:12 light dark photo period, room temperature was 22^0 C +/- 3^0 C.

Two groups of rats (n=8) were selected for the experimental procedure. The first group of rats was injected with sterile normal saline (sham vaccination, negative control). The second group was injected intraperitoneally with 10^5 colony forming units of *Pasteurella haemolytica* (experimental vaccination). Tissue was then harvested at 72 hours after exposure to either sham or experimental vaccination. Both groups were handled the same way with respect to tissue harvesting and cAMP evaluation.

B) <u>Tissue Harvesting and cAMP Measurements</u>

Each animal was anesthetized to a surgical plane of anesthesia using 80 mg/kg phenobarbital intraperitoneally. The thoracic aorta was carefully, surgically removed in its entirety and placed in Krebs-Henseleit solution (aerated with 95% oxygen and 5% carbon dioxide at 37° centigrade (+/- 1°C). Each isolated aorta was then sectioned into six approximately equally sized rings. Two of the six rings from each subject (repeated measures) were subjected to one of three drug treatments. These treatments included 3 minute exposure to one of Krebs-Henseleit solution (control treatment), isoproterenol (10^{-7} molar) or forskolin (10^{-6} molar). Each ring was gently blotted, extraneous tissue removed and weighed to the nearest milligram The tissue was then immediately frozen in liquid nitrogen and stored at -30° centigrade for subsequent evaluation of cAMP levels. The rings from each subject were matched as to their position from proximal to distal on the thoracic aorta with respect to each drug treatment.

Cyclic AMP levels were evaluated by use of a commercial cAMP enzyme-immunoassay system (Biotrack™, by Amersham Life Sciences). All tissue cyclic AMP levels were completed within 48 hours of tissue treatment.

C) <u>Preparation of Drugs and Solutions</u>

Krebs-Henseleit buffer was used during the dissection of the ex-vivo aortic rings. The composition of the Krebs-Henseleit solution (in one liter of sterile double distilled water in a sterile 5 liter Erlenmeyer flask as follows: 6.9 grams NaCl ; 0.35 grams KCl; 0.28 CaCl; 0.14 grams $MgSO_4$; 2.09 grams $NaHCO_3$; 0.16 grams KH_2PO_4; 2.0 grams dextrose) as described by Weekley (1994). This solution was aseptically mixed immediately before use.

Isoproterenol solution was prepared from stock powder (Sigma I5627) in sterile double distilled water. Isoproterenol was mixed to result in a concentration of 10^{-7} molar in a volume of 30 milliliters of Krebs-Henseleit. Each aortic ring was then exposed to this final solution for exactly 3 minutes. The solution was maintained at 37^0 centigrade (+/- 2^oC) and aerated with 95% oxygen / 5% carbon dioxide gas.

Forskolin, (Sigma, F6886) was mixed to give a final concentration of 10^{-6} molar concentration in 30 milliliters of Krebs-Henseilet. Forskolin acts to induce the activity of cAMP in the cell. The procedure for exposure of aortic rings to forskolin was identical to that of the isoproterenol.

The control solution was Krebs-Henseleit alone. Control tissues were treated in an identical manner to that described above.

D) <u>Statistical Analysis of Data</u>

This experiment is to evaluate the relative differences in cAMP concentration at three different points of the receptor to smooth muscle relaxation cascade. Each treatment result (control,

isoproterenol, and forskolin) was compared in both the vaccinated and the non-vaccinated subjects by use of the student t-test. Each assessment of cAMP was done in duplicate. The duplicated results were averaged to give a single value.

RESULTS

For each of the pharmacological treatments there is no statistically significant difference in cAMP levels between vaccinated and non-vaccinated subjects.

Comparison of control treatments in vaccinated and non-vaccinated animal failed to generate a significant difference ($p > 0.05$, figure #1). However, the results of this experiment suggests that there is a tendency for greater levels of cAMP in vaccinated subjects than in non-vaccinated subjects. This trend appears to remain consistent throughout the experimental manipulations.

The isoproterenol and forskolin treatments also failed to generate any statistically significant differences between the vaccinated and non-vaccinated groups ($p > 0.05$, figures #2 and #3). The isoproterenol treatment tended towards statistical significance. Failure to generate a significant difference using forskolin suggests a weakness in the experimental design. In future trials one must ensure experimental robustness by increasing the number of repetitions and subjects used. The limited differences in the levels of cAMP at the molecular levels and the limits of the testing procedure may necessitate higher experimental power.

Figure #1: Comparison of data between vaccinated and non-vaccinated groups exposed to the control treatment (Krebs Henseleit)

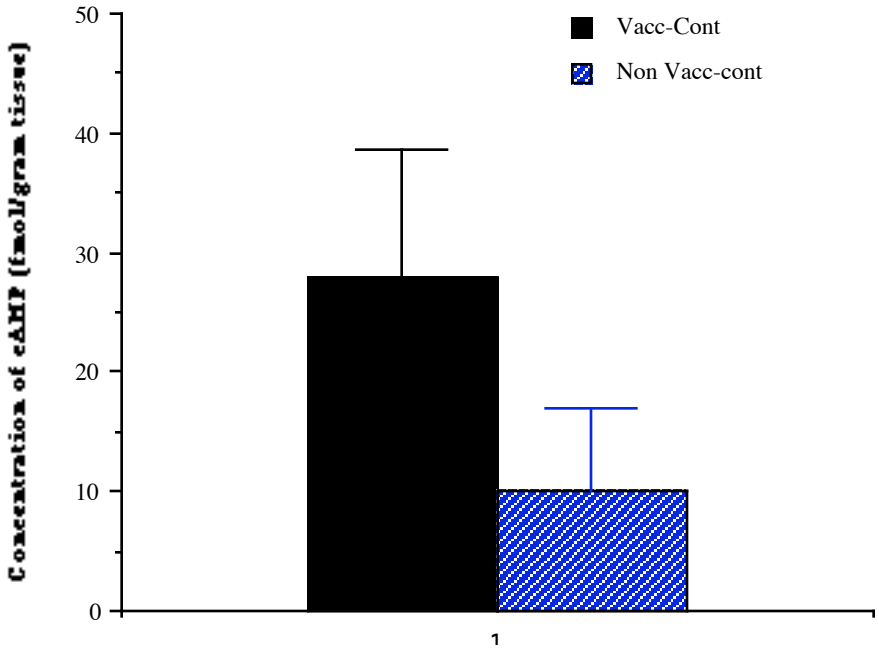

Figure # 3.1: cAMP content (fmol / microgram tissue) of rat aorta. Subjects (n=4, repeated measures design) were pre-treated with saline (control) versus PH 10^5 CFU (experimental) treatment. Data are presented as the Mean (+/- SEM). There was no sigificant difference ($p > 0.05$) between the two treatments.

Figure #2: Comparison of data between vaccinated and non-vaccinated groups exposed to isoproterenol

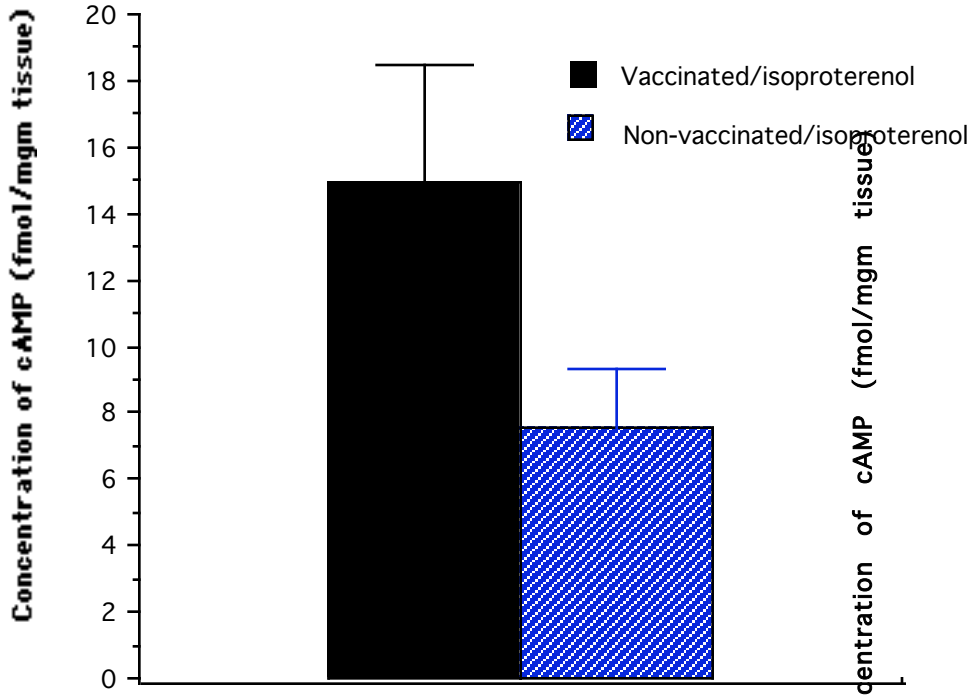

Figure # 3.2: cAMP content (fmol / microgram tissue) of rat aorta. Subjects (n=4, repeated measures design) were pre-treated with saline (control) versus PH 10^5 CFU (experimental) treatment. Data are presented as the Mean (+/- SEM). Following the harvesting of the tissue, the aortic rings were exposed to isoproterenol (10^{-7} molar for 3 minutes) then examined for the level of cAMP. There was no sigificant difference ($p > 0.05$) between the two treatments.

Figure #3: Comparison of data between vaccinated and non-vaccinated groups exposed to forskolin treatment

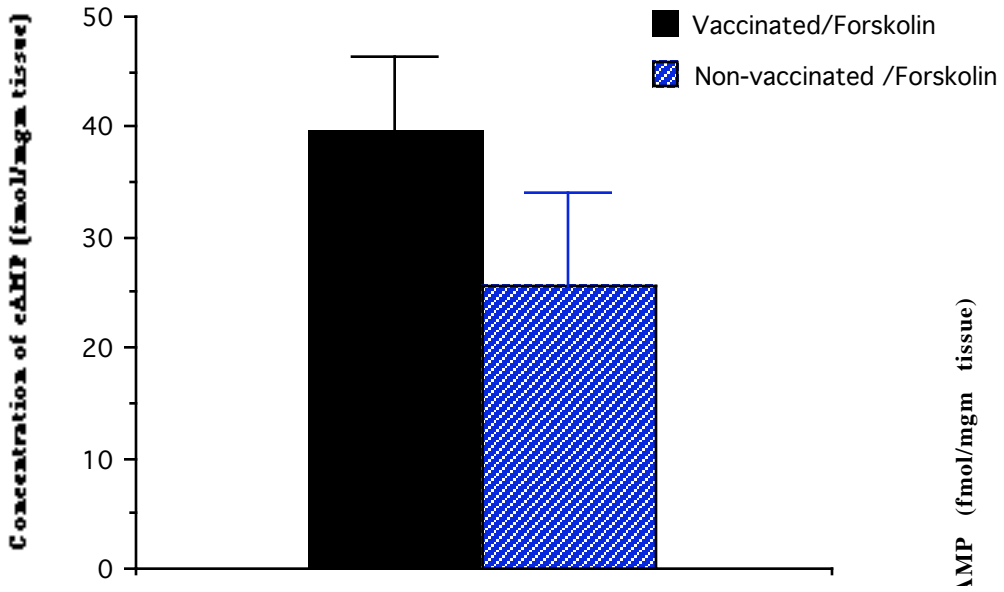

Figure #3.3: cAMP content (fmol / microgram tissue) of rat aorta. Subjects (n=4, repeated measures design) were pre-treated with saline (control) versus PH 10^5 CFU (experimental) treatment. Data are presented as the Mean (+/- SEM). Following the harvesting of the tissue, the aortic rings were exposed to forskolin (10^{-6} molar for 3 minutes) then examined for the level of cAMP. There was no sigificant difference ($p > 0.05$) between the two treatments.

DISCUSSION

Beta adrenergic receptors are integral to the homeostasis of the endothelium and vasculature in general, and the pulmonary vasculature in particular. Disruption of the BAR can explain many of the signs and symptoms associated with bovine pneumonic pasteurellosis. This line of investigation has great potential to reveal solutions with respect to the prophylactic and clinical treatment of bovine respiratory disease.

The degradation of cAMP is closely regulated. Generally cAMP is hydrolyzed to 5'-AMP by the action of cAMP phosphodiesterase. The degradation or the inhibition of a cell's ability to degrade cAMP could also lead to increased levels of cAMP.

Currently, two possible post cAPKs molecular mechanisms leading to vascular smooth muscle relaxation are proposed. The first involves the redistribution of Ca^{++} which prevents the contraction of the vascular smooth muscle (Walsh 1995; Miyashita 1997). The second mechanism involves the calcium active sites in the myosin light chain kinase. In contraction, calcium release activates the light chain myosin and initiates vascular smooth muscle contraction. The phosphorylation of the calcium active sites in the myosin light chain kinase blocks the action of the calcium molecule. This then occludes the active sites and prevents muscular contraction (Yamagishi 1994). There is a possibility that the disruption of the Ca^{++} sequestering ability of the cell is responsible for the inhibition of vascular relaxation as noted by Weekley (1993A, 1993B).

Results of these experiments are inconclusive. Though the concept and execution has merit there are likely too few subjects or repetitions to allow any conclusions to be drawn. Given the current results, expanding the number of subjects and repeating the study would, at the very least, allow for power calculations to suggest the appropriate number of subjects and repetitions.

CONCLUSIONS

1) There is not a statistical difference in the level of cAMP between vaccinated and non-vaccinated subjects.

2) Further evaluation using a larger sample size is needed before any definitive conclusions can be drawn about the level of cAMP with respect to exposure to PH specifically of endotoxin in general.

3) The evaluation of post cAMP mechanisms may be beneficial in further research of this type. Specifically evaluation of the activity of the tetramer cAMP dependent protein kinase, the cAMP phosphodiesterase activity and the level of intracellular calcium is necessary to further assess the disruption of beta adrenergic receptor mediated vascular smooth muscle relaxation.

4) Insufficient information and statistical power prevents conclusions being drawn on the data produced by this experiment.

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D). EXAMINATION OF TWO CURRENTLY USED *PASTEURELLA HAEMOLYTICA* VACCINES IN THE ASSESSMENT OF THEIR POTENTIAL FOR BETA ADRENERGIC UNCOUPLING ACTIVITY IN THE RAT AORTA

The Bovine Respiratory Disease Complex (BRDC) and specifically pneumonic pasteurellosis (also known as shipping fever or transit fever) has been recognized in cattle. This disease occurs as a series of events that are described by a complex multifactorial equation that, as yet, is not completely understood. The disease is characterized by the finding of *Pasteurella haemolytica* (PH) in the lower respiratory tract simultaneously with the clinical or pathological diagnosis of fibrinopurulent bronchopneumonia. Exposure of the host to PH alone is not always sufficient to induce the disease. To further complicate the issue, the bacterium *Pasteurella haemolytica* A-1 (PH), is a commensal found on the mucosa of the nasal and pharyngeal areas of most healthy domesticated ruminant species .

Many investigators have demonstrated that the action of respiratory viruses including: Infectious Bovine Rhinotracheitis, Parainfluenza-3 virus, Bovine Viral Diarrhea virus, and Bovine Syncytial Respiratory virus act synergistically with PH to promote pneumonic pasteurellosis. The viral component appears to disrupt the physical, cytological and immunological pulmonary defense mechanisms rendering the host more susceptible to bacterial invasion (Rosner 1971; Thomas 1974; Yates 1982; Bielefeldt Ohmann 1985; Lopez 1986; Kershen 1987; Perino 1989; Olchowy 1994). Other factors have also been found to enhance the likelihood of disease. For instance, physical stressors including: vaccination, castration, crowding, starvation, dehydration, cold exposure, and transportation may precipitate the disease (Olson 1980; Filion 1984; Slocombe 1984; Seigel 1987; Vogel 1987; Edwards 1989; Binkhorst 1990; Anderson 1991). Stress, in general, also acts to increase circulating cortisol and can result in a decrease in immunological and physical pulmonary defenses (Roth 1982; Ballieux 1987; Deitch 1987; Fisher 1989; Shaw 1990; Murata 1991).

It has been reported that prophylactic vaccination for bovine respiratory disease, particularly pneumonic pasteurellosis, is ineffective and may even compromise the vaccinated animals' health (Martin 1983; Thorlarkson 1990) . Some preliminary studies of vascular physiology with respect to vaccination sequelae were conducted by Weekley et al (1993E). The vaccines Precon® (live vaccine) and Presponse® (leukotoxoid rich, endotoxin free vaccine) administered parenterally causes both beta and alpha-2 adrenergic receptor dysfunction in ex-vivo vascular rings in rats. Similar results have been described for the pulmonary vasculature in sheep and cattle (Weekley 1991A; 1993D). Disruption of the adrenergic mechanisms due to exposure to vaccines may be associated with the perceived failure of prophylactic vaccination to meet the needs preventative health for animals. This mechanism may also be of significance in the molecular pathogenesis of the disease pneumonic pasteurellosis.

Adrenergic disruption in general and beta adrenergic uncoupling associated with the use of monovalent Pasteurella vaccines may have devastating consequences with respect to the prevention of this disease. The precise molecular mechanism and events by which vaccines act to disrupt beta adrenergic receptors has not yet been described. The vaccines evaluated by Weekley et al are not currently in use, having been replaced by new monovalent vaccines which have entered the bovine biological / vaccine market. It is reasonable to evaluate the ability of some vaccines currently in use to assess for their potential to precipitate beta receptor uncoupling. The two vaccines which were evaluated were, One Shot ® (Smith Klein Beecham) and Once PMH® (Biocor).

METHODS AND MATERIALS

A) Treatments and Subjects

Rats, adult male Sprague-Dawley (Harlan Sprague-Dawley Inc, Indianapolis, Indiana) approximately 150-175 g were used in this series of experiments. The animals initially were housed in a quarantine area for a 1 week for acclimatization to the new facility and observed for

signs of disease. The acclimatization period involved a 12:12 light dark photo period, room temperature was 22^0+/- 3^0 C.

Both One Shot® and Once PMH® were refrigerated until use as directed by the manufacturers' storage instructions. The vaccines were obtained as a sealed commercial preparation and stored at 4^0 F. Six groups of animals were used in this experiment (n=4). Three groups were controls, diluent control Once PMH® (n=4), diluent control One Shot® (n=4) and a negative sterile saline experimental control (n=4). There were also three experimental groups, One Shot® vaccine (n=4), Once PMH® vaccine (n=4), and a PH (10^5CFU) positive control (n=4). The commercial vaccines were not modified in any way prior to the use of either the diluent or vaccine.

The amount of vaccine administered for each experimental commercial vaccine was approximately 0.5 milliliter intraperitoneally. For the PH positive control group the volume was appropriate to give the necessary 10^5 CFU (approximately one milliliter). Each control animal was administered one milliliter of the appropriate control intraperitoneally.

B) <u>Preparation of Drugs and Solutions</u>

Krebs-Henseleit buffer was used in the biophysical evaluation and dissection of the ex-vivo aortic rings. The composition of the Krebs-Henseleit solution (in one liter of sterile double distilled water in a sterile 5 liter Erlenmeyer flask as follows: 6.9 grams NaCl ; 0.35 grams KCl; 0.28 CaCl; 0.14 grams $MgSO_4$; 2.09 grams $NaHCO_3$; 0.16 grams KH_2PO_4; 2.0 grams dextrose. as described by Weekley (1994). This solution was aseptically mixed immediately before use.

Potassium chloride was used for initial smooth muscle contraction. This solution was prepared in sterile, double distilled water (30 millimolar concentration when 200 microliters of stock KCl solution is added to 30 milliliters of the bathing solution).

Isoproterenol, a beta adrenergic receptor agonist, was used to evaluate the beta receptor functioning of isolated ex-vivo aortic vascular tissue. This solution was prepared in sterile, double distilled water. Serial decreasing logarithmic concentrations of isoproterenol were made from the initial 10^{-2} molar solution. The serial dilutions were made by taking 100 microliters of the initial stock solution and diluting this in 900 microliters of sterile double distilled water sequentially to 10^{-9} molar solution.

C) <u>Tissue Collection and Measurement Techniques</u>

Tissue was collected 72 hours after vaccination. Subjects were anesthetized by an intra-peritoneal administration of 80 mg/kg pentobarbital followed be the anticoagulant (25 U) of sodium heparin. Once at a deep surgical plane of anesthesia, the abdomen was opened to reveal the diaphragm. The approach to the thorax and thoracic aorta was through the diaphragm. The ventrum of the thorax was removed to completely expose the thoracic contents. Once exposed, the aorta from the base of the heart to the last rib, was carefully removed ensuring that no damage or stretching occurred. The entire vessel was then placed in warm aerated Krebs-Henseleit (37^{o}+/- 2^{o}C, 95% oxygen: 5% carbon dioxide) for further dissection and complete removal of extraneous fat and superficial tissue. All aortic rings were obtained from the base of the heart. The rings were carefully measured to be 3 mm in width. Exceptional care was taken to ensure that there was no stretching or damage to the endothelium this segment was then used for biophysical testing.

The 3 mm aortic ring, removed for biophysical testing, was then placed between two glass hooks. Biophysical testing involved the assessment of the change in tension (isometric contraction) of the aortic ring due to the addition of known concentrations of various drugs. The ring was placed between the two hooks in a thirty-five milliliter tissue bath. One hook was attached to a fixed point at the bottom of the tissue chamber while the other hook was attached to an electronic transducer that was calibrated to give output in grams tension. The tissue was placed in an insulated

heated tissue bath containing Krebs-Henseleit bicarbonate buffer . The buffer was aerated by bubbling 95% oxygen and 5% carbon dioxide through each of four individually isolated tissue baths. A constant tissue bath temperature of 37 ^{O}+/- 2^{o}C was maintained by circulating water through a temperature controlled water pump and sequentially through the outer heating jacket of each tissue bath. Each tissue ring was placed in the tissue bath under 2 grams resting tension for two hours and allowed to equilibrate. The Krebs-Henseleit buffer was replaced with fresh buffer every 15-20 minutes throughout equilibration. Testing started immediately after the equilibration period. The aortic rings, in the tissue bath, were exposed to 200 μl of potassium chloride to ensure contraction (KCl, 30 millimolar) and allowed to equilibrate to this treatment over 30 minutes. After equilibration and stabilization to KCl the recording was started and sequential increasing log molar doses of isoproterenol were added. Isoproterenol hydrochloride was used to evaluate the response of beta adrenergic receptors in mediating smooth muscle relaxation.

Electronically calibrated analog signals were transmitted from the transducer connected to the glass hook through an analog digital converter (MacLab A/D signal converter, World Precision Instruments). Digitized information was captured by MacLab data acquisition software (MacLab 2.51) on a Model M5011 Macintosh SE computer (1MB RAM, 800 K drive, 1.40 MB hard disk) in real time. Records of aortic smooth muscle relaxation as a cumulative dose response to log molar increases in the isoproterenol concentration were obtained and the results stored on a 3.5 inch computer disk.

D) <u>Statistical Analysis of Data</u>

Data were analyzed in several ways. Initially the data were compared for each experimental treatment at the isoproterenol concentration of 10^{-5} molar. In pilot studies, this concentration of isoproterenol appeared to be in the middle of the generated dose response curve and so was selected to be an appropriate point for comparison of different treatments for the experimental protocols.

The comparison was done by use of an analysis of variance (ANOVA) using group (n=4) means and standard error of the mean. This comparison was done to evaluate if there was a significant difference among treatment groups.

Further analysis consisted of a pair wise comparison of the values of 0 molar isoproterenol and 10^{-5} molar isoproterenol. Pair wise comparisons were accomplished by use of Bonferonni test. This comparison was to evaluate if there was a significant change (relaxation or contraction) with increasing logarithmic doses of isoproterenol

RESULTS

Saline IP injection was the negative PH control. The negative control resulted in significant (i.e. 0 molar isoproterenol compared to 10^{-5} molar) relaxation at the 10^{-5} molar concentration of isoproterenol concentration ($p < 0.001$). Aortic rings from PH vaccinated animals did not relax on exposure to isoproterenol. The positive control was without significant vascular, within group relaxation ($p > 0.05$). The positive controls and negative controls were significantly different from each other at 10^{-5} molar isoproterenol ($p < 0.001$, figure #1)

Both the One Shot® diluent control and vaccine experimental groups failed to show any relaxation in response to 10^{-5} molar concentration isoproterenol ($p > 0.05$). Further, there was no difference among the One Shot® experimental vaccine group, the One Shot® diluent group nor the PH positive control group ($p > 0.05$, figure #1)

Neither the Once PMH® diluent nor the vaccine in experimental animals had statistically significant within group relaxation. Those animals vaccinated with Once PMH® vaccine were not significantly different from those vaccinated with either the Once PMH® diluent alone ($p > 0.05$) or the PH positive controls vaccinated with 10^5 CFU PH ($p > 0.05$). The Once PMH® controls and experimental animals were significantly different than the PH negative controls ($p < 0.001$). These results are presented graphically in figure #2.

Fig. #1: Comparison of induction of beta adrenergic dysfunction induced by One Shot, PH (positive control) and saline (negative control).

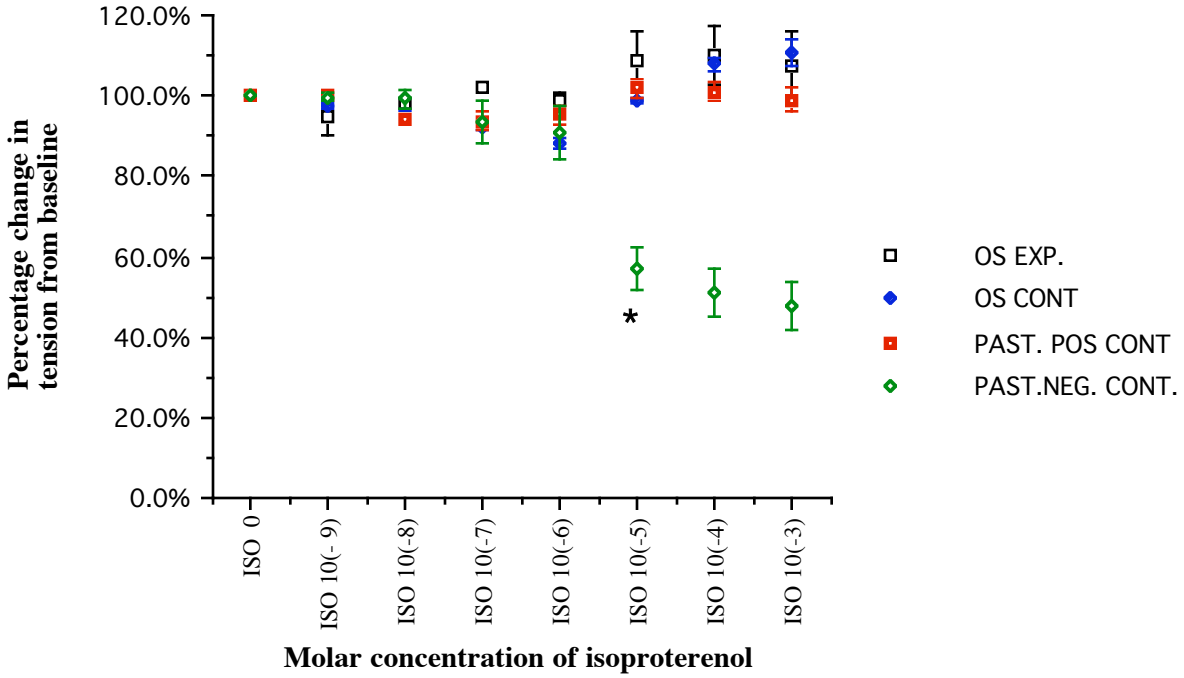

Figure # 4.1: The effect of isoproterenol HCl on vascular smooth muscle relaxation of rat aorta after pretreatment of subjects with a vaccine or PH. Subjects (n=4) were treated with either One Shot® vaccine, One Shot® control, PH (10^5 CFU, PH positive control) or saline (negative control). All treatment conditions were tested for relaxation. The positive control and both One Shot® experimental groups failed to show significant relaxation ($p < 0.05$) in response to increasing molar doses of isoproterenol. The PH negative control did however show significant relaxation at 10^{-5} molar isoproterenol ($p < 0.001$) from baseline values (*). The PH positive and negative controls were significantly different ($p < 0.001$) from each other.

Fig #2: Comparison of induction of beta adrenergic dysfunction induced by Once PMH and PH (positive control) and saline (negative control)

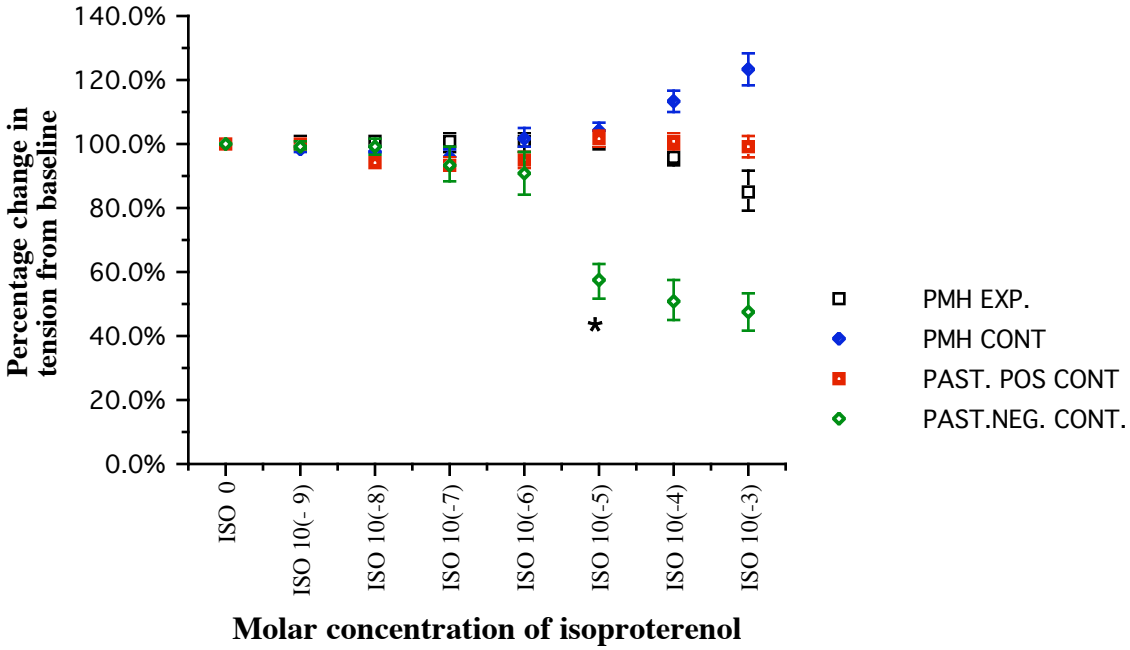

Figure # 4.2: The effect of isoproterenol HCl on vascular smooth muscle relaxation of rat aorta after pretreatment of subjects with a vaccine or PH. Subjects (n=4) were treated with either Once PMH® vaccine, Once PMH® control, PH (10^5 CFU, PH positive control) or saline (negative control). All treatment conditions were tested for relaxation. The positive control and both Once PMH® experimental groups failed to show significant relaxation ($p< 0.05$) in response to increasing molar doses of isoproterenol. The PH negative control did however show significant relaxation at 10^{-5} molar isoproterenol ($p< 0.001$) from baseline values (*). The PH positive and negative controls were significantly different ($p< 0.001$) from each other.

Overall, there was no vascular relaxation to increasing doses of isoproterenol in any group with the exception of the negative PH control (saline). All experimental and diluent control groups were statistically similar at 10^{-5} isoproterenol with respect to between group comparisons. isoproterenol caused relaxation only in the negative PH control.

DISCUSSION

The aortic rings from the positive experimental control (*Pasteurella haemolytica* 10^5 CFU) and negative experimental control (sterile saline) responded to increasing doses of isoproterenol as described by Weekley (1993E)**.** Exposure to the PH organism causes the disruption of the vascular smooth muscle beta receptor and a failure of the tissue to respond to increasing molar doses of isoproterenol. The negative experimental control (saline) doesn't appear to interfere with the pharmacological functioning of the vascular beta adrenergic receptors, as defined by this testing paradigm, indicating approximately 40% vasorelaxation.

The results of parenteral exposure of rats to One Shot ® indicates that there is a complete failure of the vascular smooth muscle to respond to isoproterenol. Similarly parenteral exposure to the diluent alone prevented the vascular beta receptors from responding to isoproterenol. This seems to indicate that there is either a chemical or biological element associated with the diluent that has the ability to inhibit beta receptors.

Once PMH® also caused disruption of the vascular beta adrenergic receptor both as the whole vaccine and as the diluent control. However, in this experiment the diluent control appeared to start to respond at higher doses of isoproterenol. At concentrations of 10^{-4} and 10^{-3} molar isoproterenol there is a distinct initiation of vasorelaxation response in the diluent control. There is statistically significant difference between the diluent and the whole vaccine at the higher doses of isoproterenol. This indicates that while there is an element in the diluent that can disrupt beta adrenergic receptors, it may not cause a complete disruption of these receptors or doesn't affect all beta receptors equally.

The mechanism by which beta adrenergic receptors are disabled is currently unknown. There is a suggestion that endotoxin may be integral to the process of beta receptor disruption.

There is however, no information concerning whether all beta receptors are affected simultaneously or even if each beta receptor is disrupted to the same extent. The results of the Once PMH® experiment suggests that, in this case, the disruption of the beta receptors may be overcome at relatively high doses of beta agonist. This may be due to the induction of partially damaged receptor mediated events or that at higher doses, undamaged receptors are recruited to result in a partial smooth muscle response to isoproterenol. The One Shot® vaccine appeared to result in total elimination of all isoproterenol mediated responses by the vascular smooth muscle. There was no response to isoproterenol at any dose examined. This suggests that there is a relationship between the dose of isoproterenol and the vascular response with respect to the pathology that initiates receptor disruption.

There is a suggestion in the literature and in a previously completed experiment in this laboratory that endotoxin may be of importance to these events (Romano 1985; Shepard 1987; Forse 1989; Campbell 1993; Bensard 1994). Endotoxin is a constituent of Gram negative bacterial cell wall that is released when the cell disintegrates. In most cases, endotoxin is easily detected by the Limulus Ameobocyte Lysate (LAL) assay but very difficult to eliminate from vaccines of gram negative organisms. In the case of commercial vaccines however, there maybe chemical entities that result in a false negative indication in the LAL (Maheswaran 1995). It has not been definitively demonstrated that endotoxin is the only constituent that can lead to vascular beta adrenergic receptor dysfunction. Further, the exact mechanism of this disruption is yet to be described, therefore the synergistic or antagonist chemical interaction can not be assessed.

Due to the proprietary nature of vaccines and biologicals it is unknown what chemical and biological additives are in the diluent or whole vaccine. It would appear from these results that at least at high isoproterenol concentrations there is a difference in the beta receptor functioning with respect to Once PMH® and One Shot®. It is unclear, at this time, if these in-vitro results occur to

the same extent in-vivo. The evaluation of this difference may suggest the mechanism or mechanisms that are involved with this phenomenon.

The vascular events associated with vaccine induced beta adrenergic receptor disruption, are of unknown clinical consequence in-vivo due to redundant protective mechanisms of the lung vasculature. Gross examination of the bovine lung tissue may indicate pulmonary congestion due the decrease in pulmonary venous flow. Interstitial edema may be present if the beta adrenergic receptors associated with mediating fluid extravasation are affected or due to increased intrapulmonary pressures secondary to lack of vasodilation. Further, should the beta receptors involved with inhibition of endothelial / coagulation events also be disrupted, one may see an increase in fibrin formation. These events are likely to occur in the immediate post-vaccination period with physiological consequences lasting up to three weeks.

The clinical consequences of the disruption of vascular beta adrenergic receptor dysfunction has not been examined. The events associated with vaccine induced dysfunction may include decreases in weight gain and food conversion, increased incidence of morbidity and mortality associated with respiratory disease in cattle and respiratory distress. Epidemiological studies may be of value in assessing these changes with respect to beta adrenergic receptor dysfunction.

The clinical value of a vaccine is its ability to protect the target population from a specific disease. The commercial usefulness of a vaccine relies on its ability to economically protect animals. It is clear that should a vaccine be effective in generating an immunoprotective response but also induce untoward effects resulting in disruption in animal growth, that vaccine is less than ideal both chemically and commercially. These results suggest that some currently used vaccines may have the ability to disrupt normal pulmonary vascular functioning. It remains to be seen if the vaccines have clinical and economic implications beyond the theoretical events presented here.

CONCLUSIONS

1) Both One Shot® and Once PMH® induce beta adrenergic receptor dysfunction. Though the actual mechanism has not been elucidated the mechanism of action is likely to involve endotoxin either in the vaccine component or in the diluent.

2) This disruption may be due to factors (e.g. endotoxin) contained in the diluent component and possibly the active portion of the vaccine. An element associated with the chemical or biological make-up of the diluent that can precipitate the disruption of vascular beta adrenergic receptors.

3) The disruption of the adrenergic receptor appears to occur to an equal extent with respect to the diluent and the whole vaccine preparation. Other vaccine preparations remain to be evaluated and may have similar effects on beta adrenergic receptors.

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E). POSSIBLE PHYSIOLOGICAL INTERACTION OF THE ALPHA-2 ADRENERGIC SYSTEM AND NONADRENERGIC-NONCHOLINERGIC (NANC) MECHANISMS OF THE RAT AORTA.

Neural control of the vascular system is essential to the homeostasis of the body. The vascular system is generally recognized to be modulated by three interactive systems that act in concert to maintain the appropriate blood flow to various organs. These systems are the cholinergic system, the adrenergic system and the nonadrenergic-noncholinergic system (NANC).

The NANC system is part of the autonomic nervous system. These neurons are described as those that fail to have the histological features of either adrenergic or cholinergic neurons of the autonomic nervous system. Many transmitter substances are associated with these neurons however nitric oxide (NO) is recognized as a very important mediator and part of the family of endothelial derived relaxing factors.

Epidemiological evidence suggests that vaccination in cattle against *Pasteurella haemolytica* can result in increases in morbidity and decreased weight gain as noted by Thorlarkson (Thorlarkson 1990) and increases in mortality noted by Martin (1980;1983). Weekley has demonstrated that, after exposure of Sprague-Dawley rats to live PH organisms, changes in the vascular smooth muscle response to alpha adrenergic agents are noted. In particular, in these experimental animals, the alpha-2 adrenergic receptor mediated system results in an exaggerated vasoconstriction response when exposed to the selective alpha-2 adrenergic agonist, clonidine. Further, the beta adrenergic agonist isoproterenol fails to elicit vasorelaxation as occurs in the untreated controls (Weekley 1993A). These deficits, as a result of the exposure of experimental animals to live PH have been demonstrated in several species including cattle, and sheep (Weekley 1991A; 1991B; 1993D; 1993E; 1994). Taken together, these results suggest an increased vasoconstriction, as a result of the disruption of the adrenergic based vasorelaxation mechanisms. This can result in ischemia in pulmonary tissue associated with the affected blood vessels. One of

the consequences may result in compromised defenses leading to an individual's increased susceptibility to respiratory disease. The implications of the experiments by Weekley et al offer a possible explanation for the vascular response noted as sequalae to prophylactic vaccination with monovalent PH vaccines in cattle.

The interaction of the vascular alpha-2 adrenergic receptor and nitric oxide of the NANC system in the homeostatic regulation of vascular tone has recently been suggested by experiments in this laboratory. In early experiments (results not presented here) it was noted that aortic rings that had not been precontracted gave the expected constriction response when exposed to clonidine. Ex-vivo aortic rings that had been exposed to the alpha-1 agonist, phenylephrine showed vasorelaxation when exposed to clonidine. These results suggest a dual nature to the response of the alpha-2 adrenergic receptor based on the predominant vascular tone. A suggestion, as a result of the possible dual nature of the alpha-2 receptors, is that very rapid relaxation responses (e.g. on the order of seconds) changes are mediated by the alpha-2 receptor. This fine vascular smooth muscle motor control in aortic rings has not previously been described.

This paper will focus on a possible explanation for the excessive vascular constriction of the alpha-2 receptors after exposure to PH A1 in-vivo, as noted by Weekley (1993B). Vascular alpha-2 adrenergic receptors are generally recognized to exist in two functionally distinct locations in the vessel. Some are directly associated with vascular smooth muscle others are associated with the vascular endothelium. The alpha-2 receptors of the smooth muscle appear to mediate smooth muscle contraction. The molecular mechanisms associated with the alpha-2 adrenergic system, leading to vasoconstriction have been studied for some time. Alpha-2 receptors act through an G-inhibitory-alpha protein that inhibits adenylylyl cyclase. This results in a decrease in intracellular cAMP levels. The mechanism by which the inhibitory G protein leads to a decrease in cAMP is unclear but is most likely associated with voltage gated calcium channels. The end result of alpha-2 adrenergic stimulation on vascular smooth muscle is vasoconstriction. More recent papers have

suggested that the mechanism, by which alpha-2 receptors mediate vasoconstriction, is dependent on the influx of extracellular calcium through voltage operated calcium channels (Parkinson 1995).

Other vascular alpha-2 adrenergic receptors are associated with the vascular endothelium. Most research has examined the functioning of these receptors with respect to the coronary vessels (Ishibashi 1997). There exists some evidence that L-arginine, a precursor of nitric oxide is involved in the vasorelaxation of the aorta in the rat (Schini 1991). The implication is that the smooth muscle may mediate vasodilation via the NANC mediator, nitric oxide. Support for this theory is given in recent papers where it been demonstrated that alpha-2 receptors can cause a nitric oxide mediated vasorelaxation in the peripheral vessels in normal rats (Brockman 1996) and in the cerebral vasculature of rats (Bryan 1995).

Nitric oxide (NO) is one of a series of molecules in the family of vascular endothelial derived relaxing factors. Nitric oxide is very short lived, on the order of seconds (Palmer 1987; Gryglewski 1988). Derived from L-arginine by the enzyme nitric oxide synthase (NOS), nitric oxide is thought to be generated by the intact vascular endothelium (Moncada 1991; Moncada 1993). Other authors have suggested that the generation and action of NO on vascular smooth muscle is independent of the need for an intact endothelium (Schini 1991). N^{w}-nitro-L-arginine methyl ester (L-NAME) is a specific inhibitor of NOS and therefore would inhibit the actions of NO in the vascular system and reduce vasodilation.

Nitric oxide released in response to the endothelial based alpha-2 adrenergic receptors which act through guanylyl cyclase to generate cyclic guanylyl mono-phosphate (cGMP) (Rapoport 1983). This cGMP then interacts with cytosolic calcium to decrease its concentration intracellularly and results in vascular smooth muscle relaxation (Fiscus 1988).

A possible explanation for the results described by Weekley with respect to the enhanced vasoconstriction observed after treatment with PH is that the NO mediated relaxation component of the alpha-2 adrenergic receptor is disrupted. The disruption in the balance of the vasorelaxation and vasoconstriction components results in an uncontrolled vasoconstriction in response to the alpha-2 adrenergic agonist clonidine. It is necessary to demonstrate that nitric oxide is mediated through alpha-2 adrenergic receptors

This paper focuses on the interaction of the adrenergic and the NANC mediator, NO, as a possible explanation for the findings reported by Weekley. This paper proposes to examine the possibility that there is an interaction between the NANC and the alpha-2 adrenergic vascular control mechanism of the rat aorta. I will examine the possibility that NO release can be mediated by clonidine through the alpha-2 receptor.

MATERIALS AND METHODS

A) Treatments and Subjects

Rats, adult male Sprague-Dawley (Harlan Sprague-Dawley Inc., Indianapolis, Indiana) approximately 150-175 g were used in this series of experiments. The animals initially were housed in a quarantine area for a 1 week for acclimatization to the new facility and observe for signs of disease. The acclimatization period involved a 12:12 light dark photo period, room temperature was 22^0+/- 3^0 C.

Three groups of rats (n=4) were selected for the experimental procedure. The first group was used to assess whether or not an alpha-1 adrenergic receptor mediated relaxation response exists in the presence of high vascular tone (positive control). High vascular tone was initiated by exposing the aortic rings in-vitro to phenylephrine a selective alpha-1 agonist. The second group

(30 minutes bathed in L-NAME) and the third group (120 minutes bathed in L-NAME) was used to asses the effect of a decrease in nitric oxide has on the alpha-2 mediated relaxation response in the presence of high alpha-1 vascular tone.

B) <u>Preparation of Drugs and Solutions</u>

Krebs-Henseleit buffer was used in the biophysical evaluation and dissection of the ex-vivo aortic rings. The composition of the Krebs-Henseleit solution (in one liter of sterile double distilled water in a sterile 5 liter Erlenmeyer flask as follows: 6.9 grams NaCl ; 0.35 grams KCl; 0.28 CaCl; 0.14 grams $MgSO_4$; 2.09 grams $NaHCO_3$; 0.16 grams KH_2PO_4; 2.0 grams dextrose). as described by Weekley (1994). This solution was aseptically mixed immediately before use.

Phenylephrine was mixed in sterile saline at the appropriate concentration to result in 10^{-6} molar in a 30 milliliter tissue bath. The phenylephrine was added to the tissue bath after tissue stabilization for 60 minutes. Five minutes after addition of the phenylephrine testing with clonidine was started. L-NAME was mixed in sterile water to result in a concentration of 10 molar in a thirty milliliter tissue bath. The tissue was first exposed to L-NAME for thirty minutes then testing initiated. The tissue was then allowed to recover and return to baseline over 20 minutes. Phenylephrine was then added for a second time (incubation for five minutes) then incubation with L-NAME for 120 minutes. The solution in the tissue bath was changed every 15 to 20 minutes pre and post-incubation.

Isoproterenol, a beta adrenergic receptor agonist, was used to evaluate the beta receptor functioning of isolated ex-vivo aortic tissue. This solution was prepared in sterile, double distilled water. Isoproterenol was mixed to result in a concentration of 10^{-7} molar in a volume of 30 milliliters of Krebs-Henseleit . Each aortic ring was then exposed to this final solution for exactly 3

minutes. The solution was maintained at 37^0 centigrade (+/- 2^oC) and aerated with 95% oxygen 5% carbon dioxide gas.

C) <u>Tissue Collection and Measurement Techniques</u>

Subjects were anesthetized with an intra-peritoneal injection of sodium pentobarbital (80 mg/kg), which was then followed by a separate but simultaneous administration of 25 U of sodium heparin. Once at a deep surgical plane of anesthesia, the abdomen was opened to reveal the diaphragm. The approach to the thorax and thoracic aorta was through the diaphragm. The ventrum of the thorax was removed to completely expose the thoracic contents. Once exposed the aorta, from the base of the heart to the last rib, was carefully removed ensuring that no damage or stretching occurred. The entire vessel was the placed in warm aerated Krebs-Henseleit (37^0 C, 95% oxygen: 5% carbon dioxide) for further dissection and complete removal of extraneous fat and superficial tissue. All aortic rings were obtained from the base of the heart. The rings were carefully trimmed to 3 mm in width. Exceptional care was taken to ensure that there was no stretching or damage to the endothelium. This segment was then used for biophysical testing.

The 3 mm aortic ring, removed for biophysical testing, was then placed between two glass hooks. Biophysical testing involved the assessment of the change in tension (isometric contraction) of the aortic ring due to the addition of known concentrations of various drugs. The ring was placed between the two hooks in a thirty-five milliliter tissue bath. One hook was attached to a fixed point at the bottom of the tissue chamber while the other hook was attached to an electronic transducer that was calibrated to give output in grams tension. The tissue was placed in an insulated heated tissue bath containing Krebs-Henseleit bicarbonate buffer . The buffer was aerated by bubbling 95% oxygen and 5% carbon dioxide through each of four individually isolated tissue baths. A constant tissue bath temperature of 37.0 +/- 2.0^oC was maintained by circulating water through a temperature controlled water pump and sequentially through the outer heating jacket of

each tissue bath. Each tissue ring was placed in the tissue bath under 2 grams resting tension for two hours and allowed to equilibrate. The Krebs-Henseleit buffer was replaced with fresh buffer every 15-20 minutes throughout equilibration and testing. Testing started immediately after the equilibration period. The aortic rings, in the tissue bath, were exposed to 10^{-6} molar phenylephrine and allowed to equilibrate to this treatment over 5 minutes. After equilibration to the phenylephrine the recording was started and sequentially increasing log molar doses of clonidine were added to evaluate the response of alpha-2 adrenergic receptors in causing smooth muscle relaxation. Aortic rings were evaluated after precontraction with phenylephrine (10^{-6} molar) without the addition of the specific NO antagonist L-NAME. This was to assess the other tissues were expressed to either 30 min L-NAME or 120 min L-NAME before assessment of the pharmacological response to clonidine in alpha-1 mediated increased vascular tone.

Electronically calibrated analog signals were transmitted from the transducer to an analog digital converter (MacLab A/D signal converter, World Precision Instruments). Digitized information was captured by MacLab data acquisition software (MacLab 2.51) on a Model M5011 Macintosh SE computer (1MB RAM, 800 K drive, 20 sc hard disk) in real time. Records of vascular smooth muscle activity, as a cumulative dose response to log molar increases in isoproterenol were obtained and the results stored on a 3.5 inch computer disk.

D) <u>Statistical Analysis of Data</u>

Data were analyzed in several ways. Initially the data were compared for each experimental treatment at the clonidine concentration of 10^{-5} molar. In pilot studies this appeared to be in the middle of the generated dose response curve and so was selected to be an appropriate point for comparison of different treatments for the experimental protocols. The comparison was done by use of an analysis of variance (ANOVA) using group (n=4) means and standard error of the mean, to evaluate for a significant difference among treatment groups.

Further analysis consisted of a pair wise comparison of the values of 0 molar clonidine and 10^{-5} molar clonidine. Pairwise comparisons were done by use of the Student "t" test. This was to evaluate for a change (relaxation or contraction) with increasing logarithmic doses of clonidine

RESULTS

Results indicate that aortic rings, in the presence of alpha-1 adrenergic increased vascular tone due to pre-contraction with phenylephrine, demonstrate vasorelaxation when an alpha-2 adrenergic selective agonist, clonidine, is introduced. The addition of clonidine, in the absence of L-NAME, and at higher doses of clonidine (e.g.10^{-3} molar) reversed the relaxation trend to result in a constriction response (figure #1).

In the presence of L-NAME, the clonidine mediated relaxation response was diminished. The reduction in vascular relaxation was proportional to the length of time the aortic tissues were exposed to L-NAME. The aortic segments treated with L-NAME for thirty minutes were significantly different than either the control group ($p< 0.01$) or the second experimental group treated for 120 minutes ($p< 0.001$). The 30 minute L-NAME group demonstrated an intermediate amount of reduction in relaxation compared to the other two groups. The group exposed to L-NAME for 120 minutes was also significantly different, less relaxation, from the control group ($p< 0.003$). The relaxation response to clonidine, appears to be diminished if, nitric oxide synthetase (NOS) and therefore nitric oxide (NO), is decreased.

Fig #1:Comparison of clonidine induced relaxation with and without the nitric oxide antagonist L-NAME

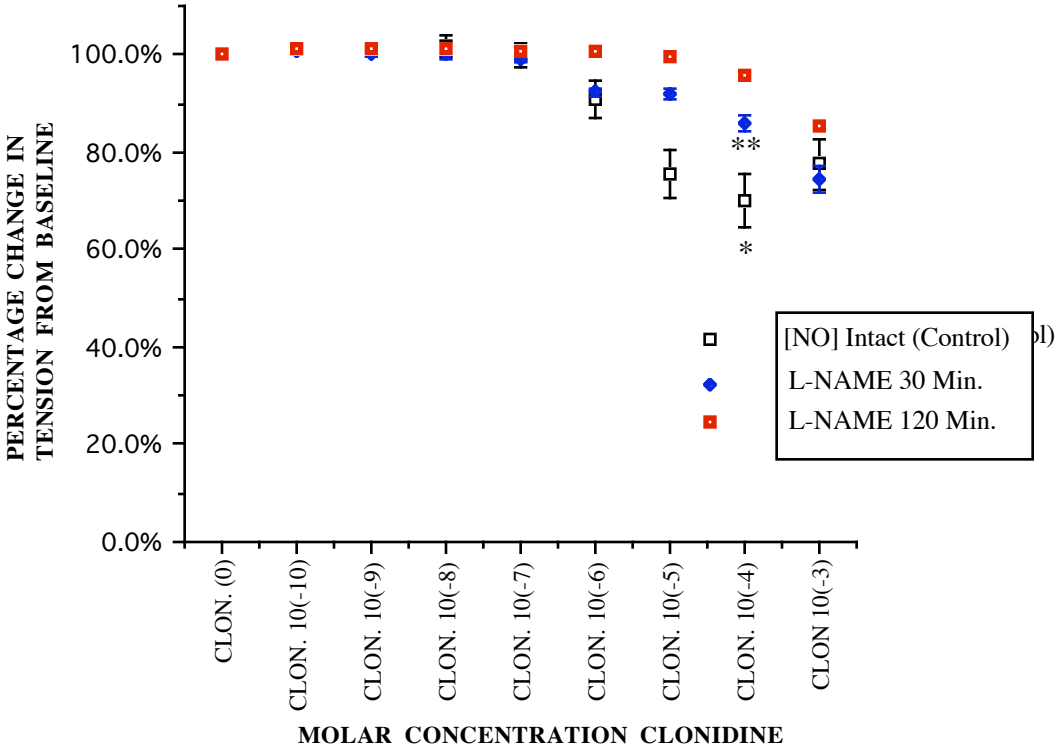

Figure # 5.1: Effect of increasing molar doses of clonidine in the presence of high phenylephrine (10^{-6} molar) induced (alpha-1) vascular smooth muscle tone. Harvested tissues from subjects (n=4) were exposed to incubation in saline (control), L-NAME 30 minutes (10 molar), L-NAME 120 minutes (10 molar). There was significant relaxation from the baseline for the control group the L-NAME 30 minute ($p< 0.01$, *) and the L-NAME 120 minute experimental group ($p< 0.001$, **).

The relaxation of the tissue for any single experimental condition (comparison of 0 molar versus 10^{-5} molar) was significant for both the untreated controls ($p < 0.001$) and the 30 minute treated experimental ($p < 0.05$). There was no significant relaxation, for a similar comparison, in the experimental group treated with L-NAME for 120 minutes ($p > 0.05$).

In both cases where L-NAME was used there appeared to be a trend towards the initiation of a relaxation response at higher concentrations of clonidine. There is a correlation between the inhibition of NOS and NO and the ability of clonidine to elicit relaxation in the presence of alpha-1 adrenergic mediated aortic tone.

DISCUSSION

Previous studies have demonstrated that the nitric oxide precursor L-arginine is sufficient to elicit relaxation of the rat aorta and that this relaxation was not dependent on the presence of an intact endothelium (Schini 1991). There is an indication from previous experimentation that both the smooth muscle cell and the endothelium can generate NO (Schini 1991). This study did not examine the interaction, at the level of the aorta, of the endothelium, alpha-2 adrenergic receptor complex and other alpha-2 adrenergic agonists. Other studies have made a connection between the stimulation of the alpha-2 adrenergic agonist and the generation of NO in the peripheral vessel, the superior mesenteric artery (Brockman 1996). In these studies, however, it was found that the endothelium is a necessary and essential feature in the generation of NO as a response to alpha-2 adrenergic receptor stimulation (Brockman 1996).

The results of this experiment suggest that clonidine can mediate vasodilation through the alpha-2 receptor resulting from an increase in nitric oxide in ex-vivo rat aorta. Nitric oxide is a very labile molecule of short duration (Palmer 1987; Gryglewski 1988). This suggests that the activity of NO is important to vascular tone on a second to second basis. The balancing of vascular tone is

a complex interaction of multiple systems. The adrenergic system has the ability to initiate vasoconstriction by the alpha-1 receptor and vasorelaxation by the activation of beta adrenergic receptors. These two adrenergic receptors work on the order of minutes. The interaction of the vascular mechanisms associated with the alpha-2 adrenergic receptors has the ability to modify vascular tone on a second to second basis.

Others have shown that there is a dynamic interaction between alpha-2 mediated vasoconstriction and vasodilation in coronary arteries in-vivo (Ishibashi 1997). This interaction is modified by the predominant vascular tone. It has yet to be demonstrated that in the in-vivo or clinical situation, the maintenance of bovine pulmonary vascular (or rat aorta) tone is a balance between the two actions of the alpha-2 receptor.

These results suggest that there is a dual nature associated with the alpha-2 adrenergic receptor. The subtype of the alpha-2 receptor, that mediates the results, noted in this paper and previous experiments, has not yet been identified and may be species and even tissue specific (Brockman 1996). It remains to be investigated as to what the effects of parenteral *Pasteurella haemolytica* A1 has on the alpha-2 adrenergic receptor mediated NO activity both as an ex-vivo preparation and as an in-vivo model.

The results of enhanced aortic constriction are consistent with the elimination of the balancing influence of alpha-2 mediated, endothelial based, NO release. There is some evidence to indicate that endotoxin (LPS) is a key element in the disruption of adrenergic receptors as demonstrated by Weekley et al (1993A; 1993B; 1994). Recent studies examining the effects of endotoxin on the alpha-2 mediated release of NO are not in agreement. Examining the effects of LPS on in-vitro cultured aortic smooth muscle cells, demonstrates that there is actually an induction of NOS and an enhancement of L-arginine transportation (Wileman 1995). Other authors have

found that LPS induces NOS in rat cardiac myocytes in-vivo (Sulakhe 1996). It is however very difficult to extend these results to the situation involving endothelial intact, ex-vivo aortic rings.

It is clear that the interactions of multiple control systems involved in the maintenance of vascular tone are complex and redundant. Furthermore LPS has direct effects on the adrenergic system, nitric oxide synthetase, nitric oxide and on the induction and regulation of the inflammatory cytokines. The affects of LPS in general and live PH specifically, remain to be investigated with respect to the alpha-2 mediated release of NO in-vivo.

CONCLUSIONS

1) The alpha-2 adrenergic receptor mediates nitric oxide relaxation that can be found in the NANC system, and is active in the ex-vivo rat aorta segments.

2) The alpha-2 adrenergic system appears to have dual regulation mechanisms that can cause both vasoconstriction and vasodilation.

3) The mechanism by which the alpha-2 adrenergic receptor causes vasodilation is associated with the molecule nitric oxide and can be blocked by incubation with L-NAME, a selective NOS antagonist.

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F). BETA ADRENOCEPTOR ACTIVITY OF MICOTIL 300® IN BOVINE PULMONARY VASCULAR TISSUE, A POSSIBLE ADJUNCT TO THERAPY

Micotil 300® (tilcomisin, 20-dexo-20-(3,5-dimethylpiperidine-1-yl) desmycosin), is a new macrolide antibiotic (Dow 1991). It has been developed to eliminate the need for multiple dosing in the treatment of bovine respiratory disease complex involving *Pasteurella haemolytica* (Merrill 1989; Gorham 1990; Morck 1993). The use of this antibiotic leads to a significant improvement in clinical signs in cattle with-in 24 hours of administration. It appears unlikely that the immediate clinical improvement seen is strictly due only to the antibiotic activity of this drug. This paper is to examine the hypothesis that Micotil 300® may have beta adrenergic activity to account for the rapid clinical improvement in cattle with BRDC.

The bovine respiratory system has a number of anatomical features that may predispose these animals to respiratory disease. The most important of these is the size of the lung in the bovine compared to body weight or metabolic activity. The ratio of the size and therefore physiological activity of the ruminant lung to gross body size is less than observed in other mammals (Veit 1978). This suggests that the bovine lung must be more physiologically and metabolically active than in other mammals to meet the same homeostatic demands (Veit, 1978). The blood supply to this organ is therefore very critical to the health of the animal and the health and homeostasis of the respiratory system. Another difference is that the bovine pulmonary vasculature has a thicker tunica muscularis than that of other mammals. The ruminant tunica muscularis can result in an enhanced constriction reaction in the presence of hypoxia to correct any ventilation-perfusion mismatches (Robinson 1984; Weekley 1995; Wren 1995) . Another possible consequence of enhanced vasoconstriction is the increased trapping or margination of leukocytes in the pulmonary vasculature, though this has yet to be investigated.

As with all mammals, the pulmonary vasculature maintains blood flow, at the capillary / alveolar level, by vasoconstriction and vasodilation. The control of the pulmonary vessels is varied and involves several neural and hormonal systems. The vascular control mechanisms include: the cholinergic, adrenergic and non-cholinergic non-adrenergic (NANC) systems among others (Breeze 1985; Barnes 1986; Barnes 1995). Despite the redundancy, diversity and integration of the systems impinging on the pulmonary vasculature, the actions of the adrenergic system appear to be of primary importance to the adequate functioning of the whole animal.

Weekley has shown in several species that the beta adrenergic mechanisms associated with the functioning of vascular smooth muscle are disrupted by exposure to parenteral *Pasteurella haemolytica* A-1 (Weekley 1991; 1991A; 1991B; 1993A; 1993B; 1993E). These findings suggest the importance of the beta receptor in mediating this disease. The distribution of beta adrenergic receptors with respect to the metabolic and physiological functions of the pulmonary vasculature suggest that the disruption of this receptor may be an integral event with respect to the pathological changes found in bovine respiratory disease complex.

Beta adrenergic receptors modulate the action of several endogenous, potentially self-injurious mechanisms at the level of the endothelium and the cell. The beta adrenergic system associated with the vascular endothelium, has been demonstrated to have a role in regulating the fluid dynamics between the intravascular and extracellular fluid compartments (Mizus 1985; Minnear 1986). Unbalanced loss of fluid to the interstitium can result in edema and decreased lung performance. Endothelial beta receptors also modify the elaboration of tissue factor, the first step of the extrinsic pathway of coagulation Tissue factor generation results in the release of thromboplastin and prothrombin which is then converted to thrombin and fibrinogen (Busso 1991). The presence of fibrin attached to the endothelial luminal surface following exposure to PH was described by Weekley and others (Majno 1961; Weekley 1993A).

The beta adrenergic receptor is an element in the modulation of leukocyte antibacterial response both in-vitro and in-vivo (Bourne 1971; Ignarro 1974; Zurier 1974; Hills 1975; Harlan 1981; Busse 1984; Engels 1985; Tecoma 1986; Warren 1987; Barnard 1992; Kubes 1993; Granger 1994; Seccombe 1994; Al-Essa 1995) . Disruption of this mechanism may have significant implications in the pathogenesis of pneumonic pasteurellosis. There is evidence for the role of neutrophils in tissue destruction. It has been demonstrated that neutrophil deficient animals suffer less pulmonary tissue damage in respiratory disease than neutrophil sufficient animals (Slocombe 1985; Breider 1991). Potentially increased pulmonary vascular margination of leukocytes due to vasoconstriction coupled with an interruption in the beta adrenergic receptor mediated inhibition of neutrophils could lead to a catastrophic release of free radicals and proteases with subsequent pulmonary and vascular tissue destruction. A similar beta adrenergic suppressive effect has been described in the macrophage with respect to the elaboration of the endogenous cytokine, Tumor Necrosis Factor-alpha (Hu 1991; Severn 1992).

Disruption of the delicate balance between BARC induced vasodilation and alpha adrenergic vasoconstriction may be important in BRDC. The consequences of beta adrenergic disruption to the pulmonary and vascular tissue may include local tissue hypoxia, further enhancing the vascular constrictive response. There would likely be extravasation of fluid, metabolic acidosis, increased trapping of leukocytes in the capillaries and post-capillary venules, and increased elaboration of cytokines, proteases and oxygen free radicals associated with neutrophils and macrophages. Reversal or amelioration of this situation may require a functional beta adrenergic mechanism.

The importance of the pulmonary vascular beta mechanism in disease has been investigated. It has been shown that the beta-2 adrenergic agonist, terbutaline, can reduce microvascular serum leakage initiated by in-vivo exposure to endotoxins (Sigurdsson 1988). This action is likely to occur by the activation of endothelial based beta receptors (Steinberg 1984; Mizus 1985; Minnear 1986; Sigurdsson 1988). There is also a reduction in the hemodynamic and respiratory effects of

endotoxin in sheep treated with terbutaline (Sigurdsson 1989). This suggests that treatment of an endotoxemic animal with a beta-2 agonist may be beneficial in alleviating the tissue damage associated with endotoxemia.

The regulatory role of beta adrenergic receptors on leukocytes in the pulmonary vasculature smooth muscle and on the vascular endothelium is established. The evidence of Weekley et al (1993E; 1994; 1995) suggests that the beta adrenergic receptor system associated with the vascular smooth muscle, may be rendered non-functional by exposure to PH. The consequences of the uncoupling of this receptor may be an important and essential step in the pathogenesis of pneumonic pasteurellosis. Further, these results suggest deficits in other beta adrenergic receptors. The pathological changes noted in bovine respiratory disease are compatible with the changes expected by disruption of both pulmonary endothelial and leukocyte based beta adrenergic receptor complexes.

The use of Micotil 300 appears to reverse the clinical respiratory signs associated with the early stages of pneumonic pasteurellosis (Merrill 1989; Gorham 1990; Morck 1993). The concept that the efficacy of Micotil 300® demonstrates some beta adrenergic activity is likely. This beta activity may be a beneficial adjunct in the treatment of pneumonic pasteurellosis. As far as is known to date beta adrenergic activity would be unique to Micotil 300® among the antibiotics commonly used for the treatment of bovine respiratory disease complex.

This paper examines the possibility that beta adrenergic receptor activity exists as part of the drug action profile of the antibiotic, Micotil 300®.

MATERIALS AND METHODS

A) Subjects

Bovine pulmonary vascular tissue was obtained from local abattoirs. The animals were of mixed breeds and gender between the ages of 0.5 and 7.0 years of age, from local sources. All animals were normal, without overt signs of disease, as assessed on antemortem and postmortem inspection. Estimated ages and weights were noted for animals involved in this study (table #1). None of the animals had received any treatments within 3 weeks of slaughter this was assessed by examination of the carcass for injection sites postmortem and review of management records. Any animals showing antemortem signs of disease, indications of medication administration or postmortem signs of disease were eliminated from this study.

B) Tissue Removal and Storage

Animals were assessed by an antemortem examination in the holding pen immediately adjacent to the abattoir. The animal was then led into a clean standing stall on the abattoir floor, stunned, rendered unconscious and insensible to pain. The animal was then bled to death by incision of the subclavian and carotid arteries and the jugular veins bilaterally. The animal was then placed in a dorsal recumbancy.

The heart and lungs were removed within 15-20 minutes. The lungs were immediately dissected for the tertiery level artery and veins. All vascular tissues were removed and placed in a Krebs Henseleit solution with-in 25-35 minutes of the animals stunning. Pulmonary arteries and veins were identified and removed. Care was taken to avoid stretching, twisting or rough handling of the tissues.

Table # 1: Subject Profiles: Distribution of sex, breed, and weight characteristics for animals used in the beta receptor identification study. All tissues were obtained from one of two abattoirs, Smith Valley Meats (SVM, Rich Creek, Virginia) or the Meat Science Teaching Laboratory at Virginia Tech (VTML)

BREED	Approx.weight (in pounds)	GENDER	AGE (in years)	SOURCE
Angus	750	Female	2	VTML
Mixed Beef	900	Male	1.5	SVM
Texas L. H.	1000	Female	3.5	SVM
Hereford	1200	Female	2.5	SVM
Angus	600	Female	.5	SVM
Jersey	325	Female	.5	SVM
Angus	750	Female	.6	VTML
Angus-Cross	900	Male	.6	SVM
Angus	1300	Male (C)	2	VTML
Charlois-Cross	850	Male (C)	1.5	SVM
Piedmont	1300	Male	1	SVM
Charlois-Cross	720	Female	1	SVM
Charlois-Cross	600	Female	1	SVM
Holstein	1200	Male	2.5	SVM
Angus-Cross	800	Male	1	SVM
Angus-Cross	1500	Male	2	SVM
Angus	1400	Female	3	SVM
Holstein	980	Male	7	SVM

The primary vessel is defined as the pulmonary vessel that leaves the heart and enters the lung parenchyma. Initially only a rudimentary removal of extravascular tissue was completed at the abattoir. Once removed the veins and arteries were placed in separate ice cooled, Krebs Henseleit solution (approximately 4^{o} C and 20-30 times the tissue volume) and labeled appropriately. The flask containing the tissue was covered and sealed with Parafilm and the entire flask placed in a ice cooler for transportation to the laboratory for testing. The time from harvesting of the tissue to testing was 3.5 hours or less.

C) Solution and Drug Preparation

Krebs Henseleit buffer was used in the biophysical evaluation and dissection of the ex-vivo aortic rings. The composition of the Krebs-Henseleit solution (in one liter of sterile double distilled water in a sterile 5 liter Erlenmeyer flask as follows: 6.9 grams NaCl ; 0.35 grams KCl; 0.28 CaCl;

0.14 grams $MgSO_4$; 2.09 grams $NaHCO_3$; 0.16 grams KH_2PO_4; 2.0 grams dextrose). as described by Weekley (1994). This solution was aseptically prepared.

Potassium chloride was used for the initial smooth muscle contraction to baseline. This solution was prepared in sterile, double distilled water (30 millimolar concentration when 200 microliters of stock KCl solution is added to 30 milliliters of the solution in the tissue bath).

Micotil 300® was prepared by dilution in propylene glycol. Serial dilutions of Micotil 300® were made by taking one hundred microliters of stock solution and diluting in 900 microliters of propylene glycol. This resulted in a stock concentration of 1.66×10^{-3} molar which was then serially diluted to give concentrations from 1.66×10^{-3} molar to 1.66×10^{-10} molar (when 100 microliters is added to 30 milliliters of the tissue bath).

Isoproterenol, a mixed beta adrenergic receptor agonist, was used to evaluate the beta receptor functioning of isolated ex-vivo aortic tissue. This solution was prepared in sterile, double distilled water. Serial decreasing logarithmic concentrations of isoproterenol were made from an initial 10^{-2} molar solution. The serial dilutions were made by taking 100 microliters of the initial stock solution and diluting this in 900 microliters of sterile double distilled water sequentially to 10^{-9} molar solution.

Terbutaline, a selective beta-2 adrenergic agonist and dobutamine, a selective beta-1 adrenergic agonist, were prepared in sterile double distilled water for each experiment. The appropriate quantity of drug was weighed out and prepared for use each day. Terbutaline and dobutamine were prepared to give a final concentration of 10^{-3} molar when 100 microliters of the stock solution is added to a 30 milliliter tissue bath. Serial dilutions of the stock solution were made from 10^{-3} to 10^{-10} molar for each drug used in the experiment.

The antagonists were prepared in a similar manner as the other drugs Atenolol, a selective beta-1 antagonist and ICI118,551, a selective beta-2 antagonist, were also prepared using sterile double distilled water. Each drug was prepared to give a final concentration of 10^{-6} molar in a 30 milliliter tissue bath from the stock solution. Each drug was made freshly on the day of its use.

The vascular samples for the cumulative dose-response experiment were placed in one of three experimental groups. One set of vascular rings, acting as a standard, (assayed with known adrenergic agents, isoproterenol, terbutaline or dobutamine) was compared to the experimental tissues (assayed with Micotil 300®). Non-specific beta receptor activation (isoproterenol) was then compared to the relative contributions of the beta-1 (dobutamine) and beta-2 receptors (terbutaline). Tissues were then evaluated in the presence of a selective beta-receptor blocking drugs propranolol (mixed), atenolol HCl (beta-1) and ICI-118,551 HCl (beta -2).

D) Testing Protocol

Once in the laboratory, the vascular tissue samples were carefully removed from the transportation/storage flasks. The tissue was allowed to warm to room temperature (approximately 22° C), then was placed in a tissue bath of warmed Krebs-Henseleit solution (37° +/- 2.0°C) that had been aerated with oxygen and carbon dioxide (95% O_2 / 5% CO_2). Extravascular tissue was removed and two 3 mm wide vascular rings were removed from each vessel. Two samples of each pulmonary artery and vein from the same animal, were run in separate tissue baths under identical conditions. The prepared vascular segments were mounted on glass hooks and suspended between an electronic transducer (Grass Model #FT-103) and an anchor point in the tissue bath (30 mls) aerated with 95% oxygen / 5% carbon dioxide gas. The tissues were allowed to equilibrate at 2 grams tension for 1.5 to 2 hours. A baseline reading was then obtained to ensure tissue stability. The tissue was then pre-contracted with 30 millimolar potassium chloride and this level of contraction was defined as baseline (100% contraction). Various standard agents, dobutamine,

terbutaline and isoproterenol and the experimental agent Micotil 300® were used to induce relaxation, while propranolol HCl and selective beta antagonist agents (ICI 118,551 and atenolol) were used to inhibit the various subtypes of beta adrenergic receptors. Cumulative dose response curves were generated by the addition of sequentially increasing log doses of the appropriate agonists in the absence and presence of respective antagonists. The cumulative dose response information of vascular smooth muscle change in tension was collected using the digitally based "MacLab" data collection system and stored on 3.5 disks. Each tissue bath was drained and the Krebs-Henseleit was replaced every fifteen to twenty minutes throughout the experimental period to ensure that there was no build up drugs and metabolic waste products.

E) <u>Statistical Evaluation</u>

Data were analyzed in several ways. Initially analysis consisted of a pair wise comparison of the values of 0 molar and 10^{-5} molar within each treatment (for each agonist). These data assessed whether any change was statistically significant. The data were then compared between treatments at the drug concentration of 10^{-5} molar. The comparison between and within groups was done by use of analysis of variance (ANOVA, n=4). This comparison was done to evaluate if there was a significant difference among treatment groups. Post-ANOVA pairwise comparisons were done by the Bonferonni test of specific selected groups. This comparison evaluated the difference for a challenge with each agonist alone and in the presence of an antagonist.

RESULTS

Micotil 300®caused relaxation in the bovine pulmonary vessels (figures #1 and #2). The change in tension, relaxation, from the baseline is significantly greater in the pulmonary vein than in the artery ($p< 0.01$). The trend to relaxation from the baseline, in the artery, fails to reach statistical significance ($p> 0.05$). The question as to whether relaxation is mediated by a beta receptor was addressed by attempting to block the Micotil 300® induced relaxation by the addition of propranolol. The dose of propranolol added to each tissue bath was 10^{-6} molar, which resulted in an elimination of the relaxation response from baseline, in the case of the pulmonary vein ($P> 0.05$, no significant change between the baseline and 10^{-5} molar Micotil 300®). A trend to antagonism of the relaxation response was noted for the pulmonary artery, however, Micotil 300® failed to induce vascular relaxation at a level significantly different from baseline. It is therefore difficult to assess the true significance of the extent of the inhibition, by propranolol, of the relaxation in the pulmonary artery. Comparison of the difference in the relaxation noted in the vein with Micotil 300® (at 10^{-5} molar Micotil 300®) in the presence of propranolol (at 10^{-5} molar Micotil 300®) also yielded a significant differences between groups ($p< 0.01$)

To assess the subtypes of beta adrenergic receptors involved in this response, selective beta receptor agonists and antagonists were employed. These drugs were used to elucidate the relative contributions of each receptor subtype involved in the relaxation response for each tissue.

Assessment of the of beta-1 receptor component of the pulmonary vascular relaxation response to Micotil 300® yielded some interesting results. The pulmonary artery responded with significant relaxation to dobutamine ($p< 0.001$) which was not blocked by atenolol (figure #3). The assessment of the ability of atenolol at 10^{-6} molar concentration to block the dobutamine response suggests that the concentration of atenolol was insufficient to antagonize dobutamine. Other explanations include that that there is a displacement of the antagonist by the agonist, or that the

number of subjects is too small. The vein did not relax in response to increasing molar concentrations of dobutamine ($p > 0.05$, figure #4). A comparison of the relative relaxation induced by dobutamine in both the artery and vein showed a greater relaxation response in the artery than in the vein (figure #5). This difference in response to dobutamine between the two tissues was statistically significant ($p > 0.01$).

Evaluation of the beta-2 adrenergic receptor in the relaxation response was examined. Terbutaline failed to cause significant relaxation in the pulmonary artery ($p > 0.05$, figure #6). Further, there was no significant difference between tissues with and without ICI 118,551 ($p > 0.05$). There was however, significant relaxation induced by terbutaline in the pulmonary vein ($p < 0.01$, figure #7). No significant relaxation occured in response to terbutaline in the presence of ICI 118,551 ($p > 0.05$). This indicates that the relaxation response to terbutaline was blocked by the selective beta-2 antagonist, ICI-118,551 (at 10^{-6} molar concentration, $p < 0.01$ examined at 10^{-5} molar terbutaline with and without ICI 118,551).

Graphical comparison of the pulmonary artery and vein, suggests that the vein is more responsive to the relaxation effect of Micotil 300® than the artery (figure #9)

The ability of the selective beta-2 antagonist, ICI 118,551, to block the relaxation response of Micotil 300® in pulmonary venous tissue, was examined. The pulmonary vein did not relax to Micotil 300® in the presence of ICI 118,551 ($p > 0.05$, figure #8). Examination of the effect of Micotil 300® alone and Micotil 300® in the presence of ICI 118,551 suggests that there is a significant difference when these groups are compared ($p < 0.01$, figure #9).

The assessment of the potential of the beta-1 antagonist to directly block the action of Micotil 300® was examined. In the vein the atenolol failed to block relaxation induced by Micotil 300® (figure #10). There was a significant difference between the baseline and 10^{-5} molar Micotil

300® in the presence of atenolol however there was not a significant difference between the amount of relaxation to Micotil 300® in the presence and in the absence of atenolol ($p > 0.05$) compared at 10^{-5} molar concentration of Micotil 300®.

DISCUSSION

The results of these experiments indicate that Micotil 300® the commercially available form of tilmicosin phosphate, causes relaxation in the bovine pulmonary vascular tissue and that this relaxation is mediated by beta adrenergic receptors. The clinical impressions with respect to the use of Micotil 300® suggests immediate improvement (within 24 hours) following a single intramuscular injection. This is particularly true when Micotil 300® is used early in the course of BRDC.

Micotil 300® causes greater relaxation in pulmonary veins than pulmonary arteries. There is a trend towards relaxation in the bovine pulmonary artery but this fails to reach significance. The artery contains a higher percentage of vascular smooth muscle and elastic tissue relative to the total tissue weight as a compared to the vein. The development of the mature vascular tissue and therefore its response to pharmacological agents may vary with the breed, sex, age and immunological history. The relative density of beta-1 versus beta-2 receptors is clearly of importance in this disease. These factors could play a greater role in the development of arterial tissue as compared to venous tissue. This may account for the differential response of the tissue to Micotil 300®. Unfortunately these factors were beyond the control or assessment of this study.

Another possible explanation of the variability seen in the arteries may relate to a mixed population of beta adrenergic receptors. This study gives some evidence to support the

Figure #1: Comparison of Micotil induced relaxation in the bovine pulmonary artery with and without propranolol.

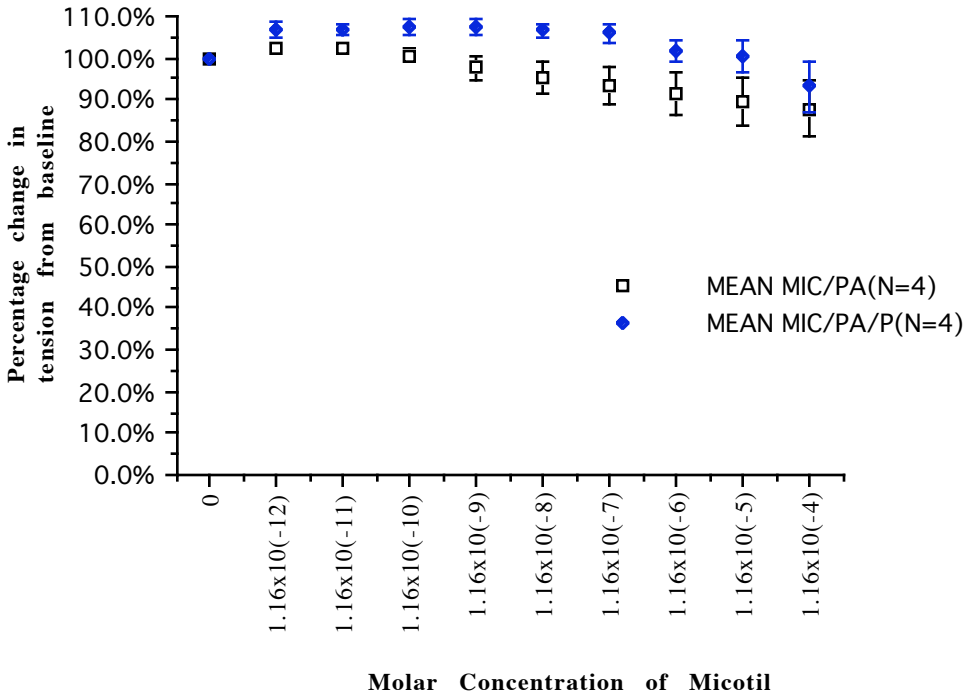

Figure # 6.1: Response of arterial smooth muscle to increasing molar concentrations of Micotil 300® with (MIC/PA/P) and without (MIC/PA) propranolol. Data are presented as the Mean (+/- SEM, n=4). Assessment of the contribution of beta adrenergic receptors in the relaxation of pulmonary arterial smooth muscle was graphed. Relaxation from baseline by Micotil 300® of the pulmonary artery was not significant ($p > 0.05$). Propranolol was unable to reduce relaxation as there was no significant relaxation with Micotil 300®.

Figure #2: Comparison of Micotil induced relaxation in the bovine pulmonary vein with and without propranolol.

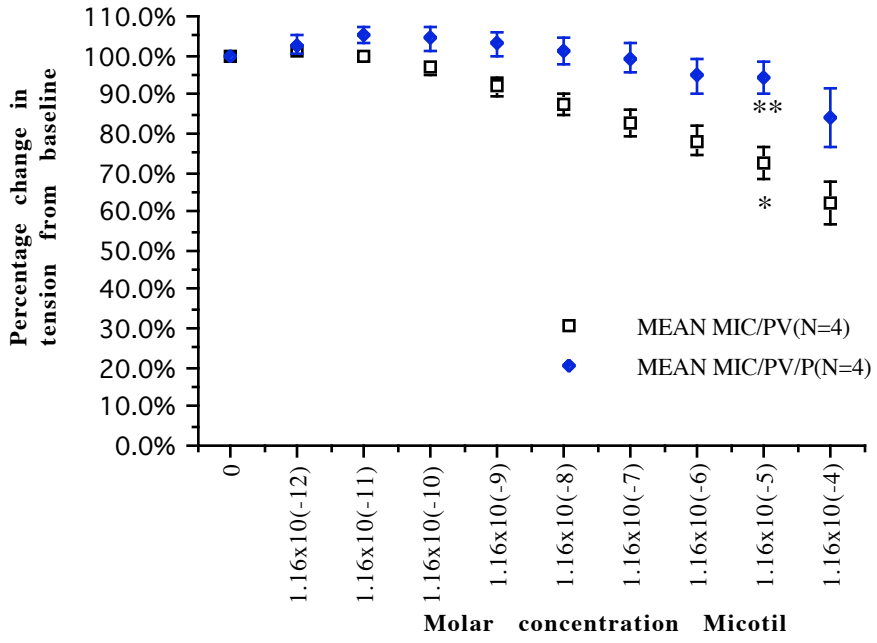

Figure # 6.2: Response of venous smooth muscle to increasing molar concentrations of Micotil 300® with (MIC/PV/P) and without (MIC/PV) propranolol. Data are presented as the Mean (+/- SEM, n=4). Assessment of the contribution of beta adrenergic receptors in the relaxation of pulmonary venous smooth muscle was graphed. Relaxation from baseline by Micotil 300® of the pulmonary vein was significant ($p > 0.001$, *). Propranolol did prevent significant ($p > 0.01$, **) relaxation from the baseline.

Figure #3: Comparison of dobutamine induced relaxation on the bovine pulmonary artery with and without atenolol.

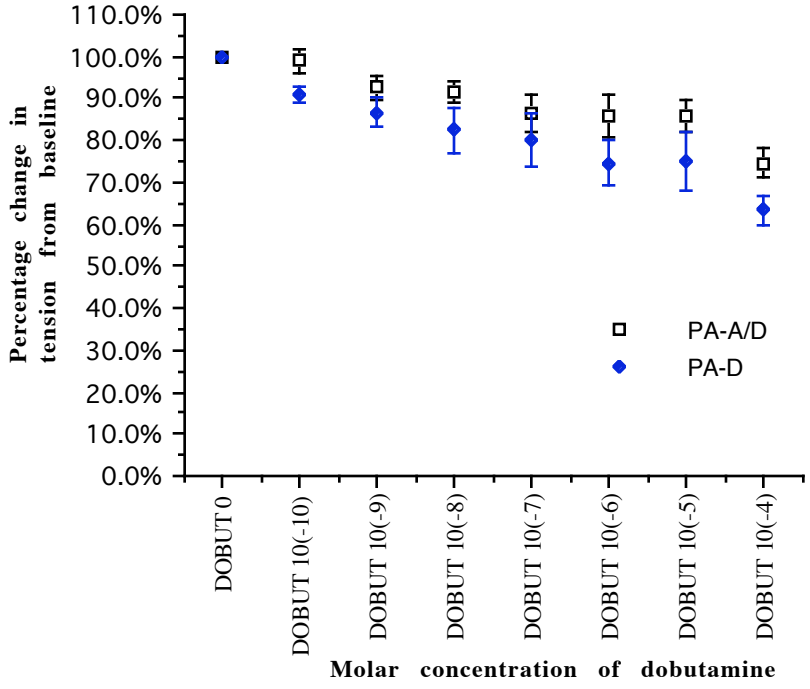

Figure # 6.3: Response of arterial smooth muscle to increasing molar concentrations of dobutamine with (PA-A/D) and without (PA-D) atenolol. Data are presented as the Mean (+/- SEM, n=4). Assessment of the contribution of beta-1 receptors in the relaxation of pulmonary arterial smooth muscles was graphed. Relaxation from baseline by dobutamine of the pulmonary artery was significant ($p > 0.001$, *). Atenolol did not prevent significant ($p > 0.05$) relaxation from the baseline.

Figure #4: Comparison of dobutamine induced relaxation of the bovine pulmonary vein with and without atenolol

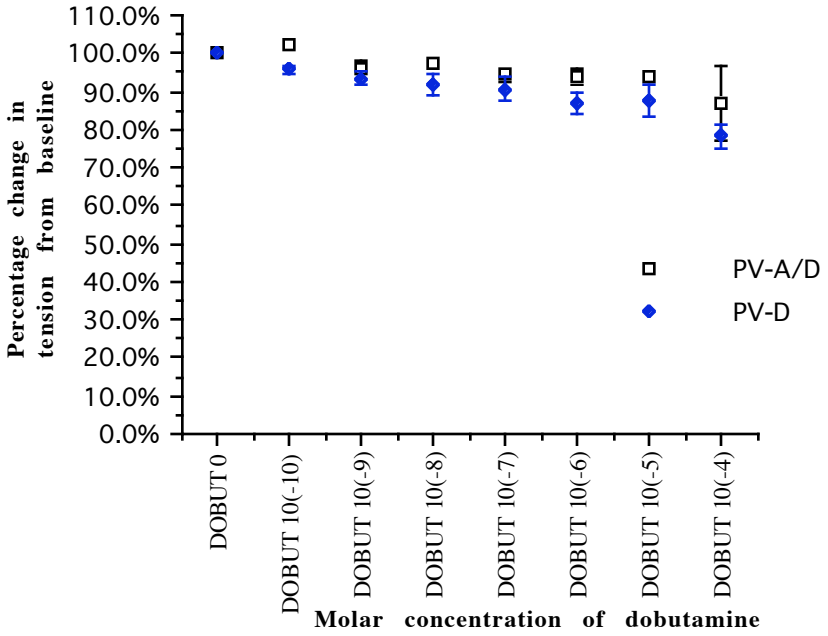

Figure # 6.4: Response of venous smooth muscle to increasing molar concentrations of dobutamine with (PV-A/D) and without (PV-D) propranolol. Data are presented as the Mean (+/- SEM, n=4). Assessment of the contribution of beta adrenergic receptors in the relaxation of pulmonary arterial smooth muscles was graphed. There was no significant relaxation from baseline by dobutamine in the pulmonary artery ($p > 0.05$). Atenolol was unable to reduce relaxation as there was no significant relaxation with dobutamine.

Figure #5: Comparison of dobutamine induced relaxation in the bovine pulmonary artery and vein.

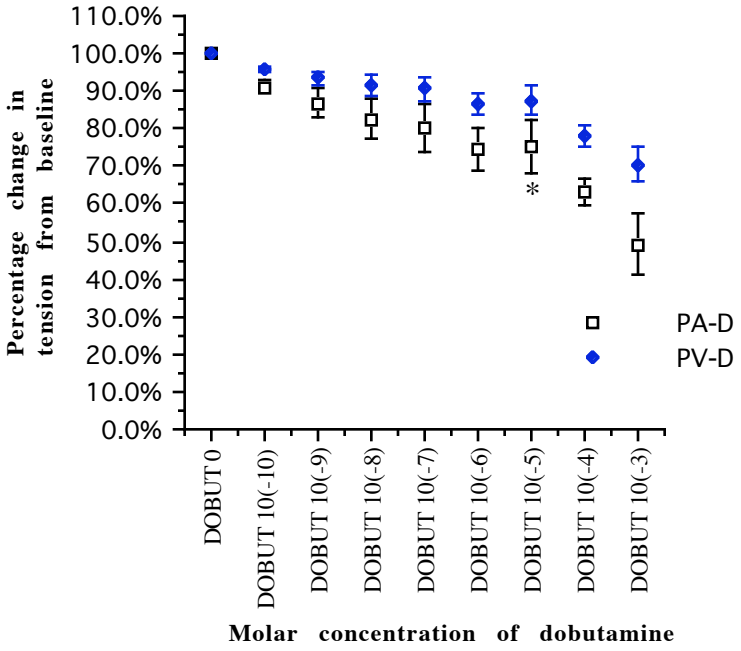

Figure # 6.5: Comparison of the response of vascular smooth muscle to increasing molar concentrations of dobutamine. Data are presented as the Mean (+/- SEM, n=4). Assessment of the contribution of beta -1 adrenergic receptors in the relaxation of pulmonary arterial (PA-D) and venous (PV-D) tissue was graphed. There was significantly more ($p < 0.01$, *) relaxation in the arterial tissue than in venous tissue.

Figure #6: Comparison of terbutaline induced relaxation in the bovine pulmonary artery with and without ICI 118,551.

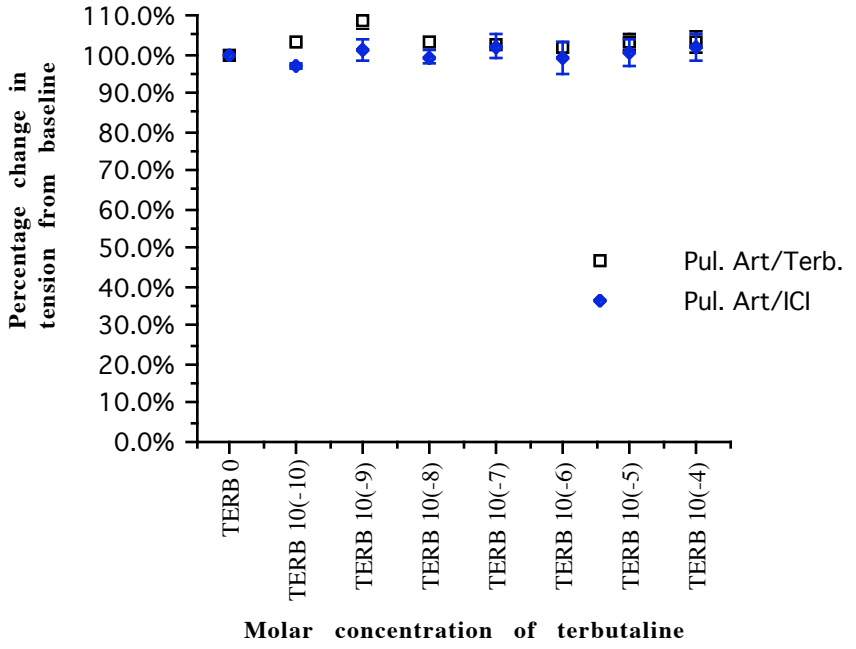

Figure # 6.6: Response of arterial smooth muscle to increasing molar concentrations of terbutaline with (Pul Art/ICI) and without (Pul Art/Terb) the antagonist ICI 118,551. Data are presented as the Mean (+/- SEM). Assessment of the contribution of beta-2 adrenergic receptors in the relaxation of pulmonary arterial smooth muscles was graphed. There was no significant relaxation from baseline by terbutaline ($p > 0.05$) in the pulmonary artery. ICI 118,551 was unable to reduce relaxation as there was no significant relaxation with dobutamine.

Figure #7: Comparison of terbutaline induced relaxation in the bovine pulmonary vein with and without ICI 118,551.

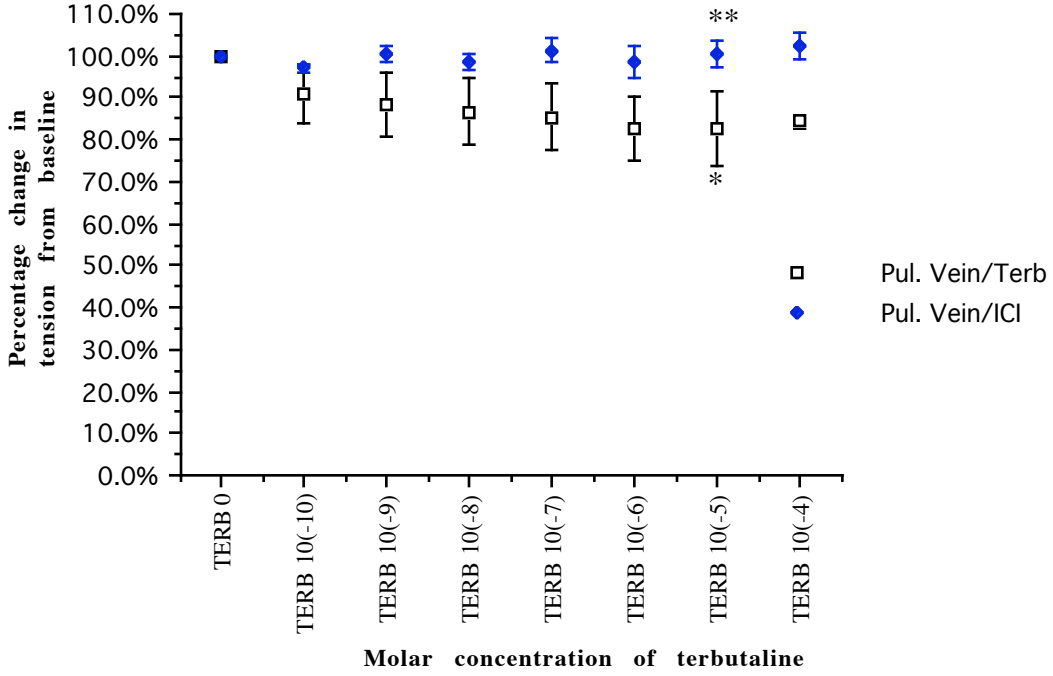

Figure # 6.7: Response of venous smooth muscle to increasing molar doses of terbutaline with (Pul Vein/ICI) and without (Pul Vein/Terb) the antagonist ICI 118,551. Data are presented as the Mean (+/- SEM, n=4). Assessment of the contribution of beta-2 adrenergic receptors in the relaxation of pulmonary venous smooth muscles was graphed. There was significant relaxation from baseline by terbutaline ($p < 0.01$,*) in the pulmonary vein. There was no significant relaxation in the presence of ICI 118,551 ($p > 0.05$, **) in the pulmonary vein.

Figure #8: Comparison of terbutaline induced relaxation in the bovine pulmonary artery and vein.

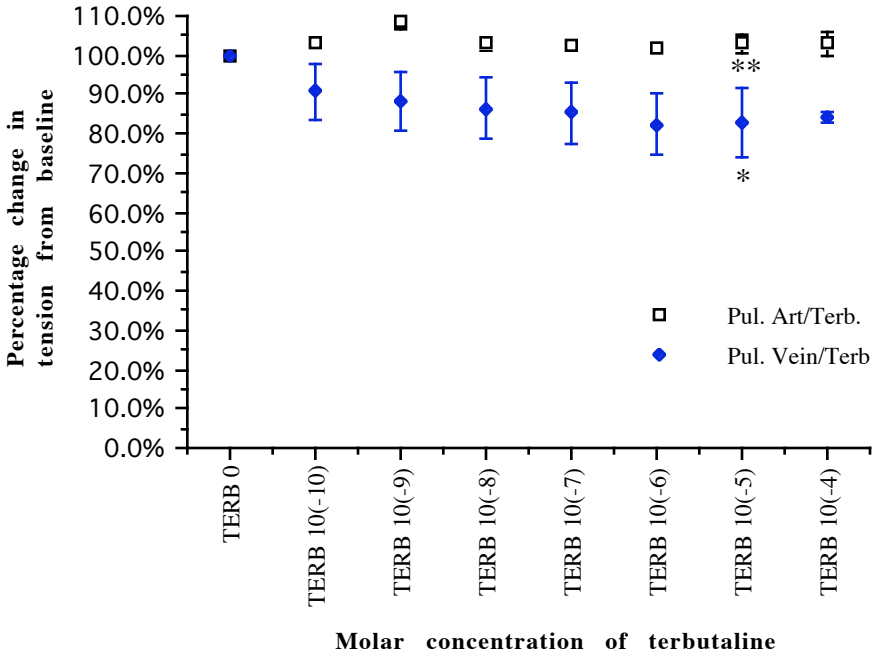

Figure # 6.8: Response of venous smooth muscle to increasing molar concentrations of terbutaline on the pulmonary artery (Pul Art/Terb) and pulmonary vein (Pul Vein/Terb). Data are presented as the Mean (+/- SEM, n=4). Assessment of the contribution of beta-2 adrenergic receptors in the relaxation of pulmonary artery versus vein was graphed. There was no significant relaxation in the arterial tissue from baseline by terbutaline ($p > 0.05$), however there was significant relaxation in the venous tissue ($p < 0.01$,*). The relaxation responses of the artery and vein were significantly different ($p < 0.01$, **).

Figure #9: Comparison of Micotil induced relaxation in the bovine pulmonary vein with and without ICI 118,551.

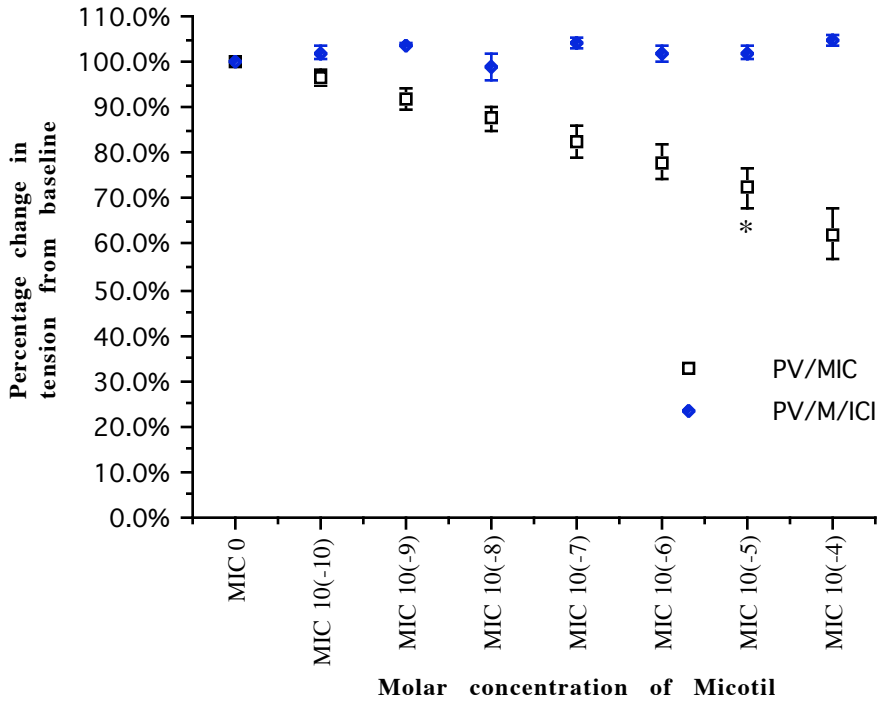

Figure # 6.9: Response of vascular smooth muscle to increasing molar concentration of Micotil 300® with (PV/M/ICI) and without (PV/MIC) the beta-2 adrenergic selective antagonist ICI 118,551. Data are presented as the Mean (+/- SEM, n=4), Assessment of the contribution of beta-2 adrenergic receptors in the relaxation of pulmonary venous smooth muscles was graphed. There was significant relaxation from baseline by Micotil 300® ($p > 0.001$,*) in the pulmonary vein. ICI 118,551 was able to reduce the Micotil 300® induced activity as there was significant relaxation from baseline ($p > 0.05$).

Figure #10: Comparison of Micotil induced relaxation in the bovine pulmonary vein with and without atenolol.

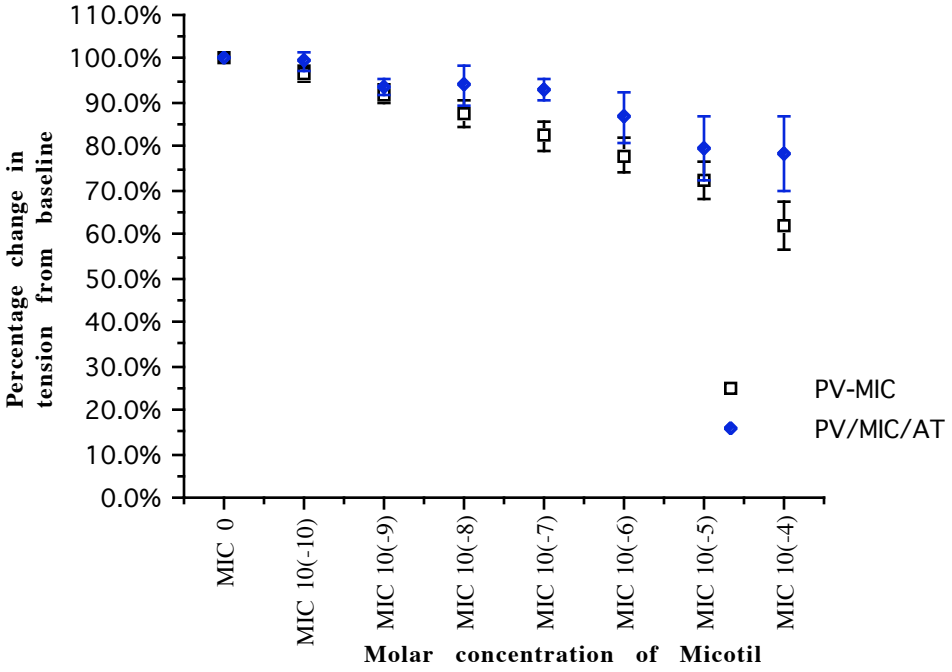

Figure # 6.10: Response of venous smooth muscle to increasing molar doses of Micotil 300® with (PV/MIC/AT) and without (PV/MIC) the beta-1 adrenergic selective antagonist atenolol. Data are presented as the Mean (+/- SEM, n=4). Assessment of the contribution of beta-1 adrenergic receptors in the relaxation of pulmonary venous smooth muscle was graphed. There was significant relaxation from baseline by Micotil 300® ($p > 0.001$,*) in the pulmonary vein. Atenolol was unable to reduce the Micotil 300® induced activity ($p > 0.05$).

claim that the beta 1 and beta 2 adrenergic receptors exist in the bovine pulmonary artery with the beta-1 receptor being predominant. The vein very likely also contains a mixed population of beta receptors where the beta 2 receptor is the dominant. The evidence presented here is that a mixed population of beta 1 and beta 2 receptors exist on both the venous and arterial sides of the pulmonary vasculature. Further data suggest that Micotil 300® has greater activity at the beta 2 receptor. The specific differential activity among beta adrenergic receptors has yet to be quantified.

It is clear that the venous aspect of the pulmonary vasculature responds more vigorously to Micotil 300® induced vasorelaxation than the arterial tissue. This could result in increased venous drainage. The significance of these findings with respect to the overall disease process is only conjecture at this time. The disruption of beta adrenergic vascular receptors after exposure to *Pasteurella haemolytica* A1 appears to occur over a 24 hour period with only partial disruption in the first 24 hours. The obvious consequence of deficient beta receptors in the balancing equation between alpha receptor contraction and beta mediated relaxation is a predominence of the alpha receptor mediated vasoconstriction. Therefore any decrease in pulmonary congestion, secondary to an up-regulation of beta mediated relaxation may be of benefit to the animal afflicted with BRDC.

The significance of decreased venous drainage in the presence of a relatively unchanged arterial supply is obvious. The pathogenesis of BRDC in general, and pneumonic pasteurellosis in particular, is recognized as fibrinous, congested lung fields. The action of a specific venous dilator, Micotil 300®, may be to activate those beta receptors that are not affected early in the disease process. This may serve to recruit additional beta adrenergic receptors which would assist in the reduction of the pulmonary congestion noted in bovine pasteurellosis. This may be the explanation for the immediate relief of signs in cattle diagnosed early with BRDC following the use of Micotil 300®. This theory is not without foundation, as Sigurdsson (1988) has demonstrated that the use of the beta-2 adrenergic receptor agonist, terbutaline is effective in relieving the symptoms of respiratory distress in sheep exposed to endotoxin.

The beta adrenergic mechanism, though important in the maintenance of various pulmonary vascular homeostatic mechanisms, has largely been ignored with respect to the etiology andtreatment of BRDC. Weekley has shown that the activity of PH administered parenterally, in multiple species can disrupt beta adrenoceptors associated with vascular smooth muscle (Weekley 1991A; 1993A; 1993E)..

The disruption of beta adrenergic receptors is unlikely to be limited to those receptors associated with vascular smooth muscle. The maintenance of the pulmonary vascular integrity is intimately involved with beta receptors associated with the endothelium. Other beta adrenergic receptors act to modulate extravasation of intravascular fluid and osmotic elements (Mizus 1985; Minnear 1986) and inhibit endothelial reactions with the coagulation cascade, thereby preventing the generation of intravascular fibrin. Still other beta receptors act to modulate the release of antibacterial enzymes and oxygen free radicals by neutrophils and cytokines by macrophages in the presence of bacteria or bacterial products. Though not yet specifically investigated, the theoretical consequence of the disruption of these beta receptors or a partial disruption of the populations of beta receptors associated with the pulmonary vascular system may account for the majority of the gross and microscopic pathological findings associated with pneumonic pasteurellosis.

The macrolide antibiotic, Micotil 300® has exceptional efficacy in the treatment of bovine pneumonic pasteurellosis. This antibiotic also appears to have significant beta adrenoceptor activity which may account in part for its clinical efficacy. It is apparent that the bovine pulmonary vasculature examined contains a mixed population of beta receptors with predominant features of the beta-1 receptor on the arterial side and beta-2 receptor on the venous side. It is likely that the predominant beta-2 adrenergic receptor activity of Micotil 300® has a beneficial effect early in the course of pneumonic pasteurellosis, to reduce the severity of pulmonary congestion. It may further be speculated, based on the homeostatic activities of the vascular beta adrenergic receptors, that beta-

2 adrenergic activity will also reduce extravasation of fluid, decrease endothelial elaboration of coagulation active products and inhibit release of enzymes and oxidative free radicals from leukocytes.

CONCLUSIONS

1) Micotil 300® has the ability to cause vasorelaxation in pulmonary vasculature.

2) This vasorelaxation is more prominent in the pulmonary veins than in the pulmonary arteries.

3) The veins appear to respond to beta-2 adrenergic receptor agonists while the arteries appear to respond more vigorously to beta 1 adrenergic agonists in-vitro.

4) These results when combined with the differential sensitivity for vasorelaxation between the pulmonary veins and arteries suggests that Micotil 300® has a predominant beta 2 adrenergic receptor activity.

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IV. GENERAL DISCUSSION

The importance of beta adrenergic receptor activity to the pathogenesis of Bovine Pneumonic Pasteurellosis, and possibly vaccine induced immunopathology have not yet been demonstrated in the target species. The accumulating evidence is providing growing evidence that the adrenergic system in general and the beta adrenergic receptors in particular are intimately involved in the development of Bovine Respiratory Disease Complex and pneumonic pasteurellosis.

Intimate involvement of the adrenergic system associated with the pulmonary venous system is physiologically well described. The beta adrenergic receptor has been demonstrated to be involved with vascular smooth muscle control, endothelial control of thrombolic events and extravasation of intravascular fluid and finally control of the elaboration of cytokines, elastase and oxygen free radicals in cells. The pulmonary alveolar and intravascular macrophages as well as the neutrophil are examples of immunologically important cells controlled by the BARC.

Beta adrenergic receptor mechanisms may be altered, as defined by its lack of response to selective pharmacological agents, after exposure to PH. Previous researchers have demonstrated that the exposure to PH as in the form of a monovalent vaccine can have deleterious effects on the adrenergic vascular smooth muscle system. It remains to be seen what the affect of exposure to PH would be in a method consistent to a field situation in the target species. However, the current experiments suggest that the vascular smooth muscle associated beta adrenergic receptor / transduction mechanism is deleteriously affected.

Experimental results indicate that exposure to parenteral PH, in the form of a vaccine, can cause a pharmacologically defined beta adrenergic disruption for as long as twenty one days post - exposure. This disruption appears to occur within 24-48 hours of exposure. Though this is not

representative of the classical field situation it speaks to the need for early intervention in the treatment of pneumonic pasteurellosis in cattle.

The establishment of the affects of endotoxin on cardiac beta adrenergic receptor mechanisms has been well established . There is now some evidence that endotoxin alone may also affect rat aortic vascular associated beta adrenergic receptors mechanisms. The recognition that endotoxin is the culprit gives some insight into future research in the development of vaccines to prevent this economically devastating disease.

Evidence from experiments examining the level of cAMP following PH exposure fail to meet scientific or experimental criteria to glean any conclusions. Perhaps increasing the number of experimental units would add sufficiently to the experimental power as to allow conclusions to be drawn.

Investigation of the alpha-2 receptor demonstrates that the receptor complex is associated with vasorelaxation through the mechanism defined by cGMP as the second messenger and mediated by nitric oxide. This suggests that cGMP of the alpha-2 receptor mechanism is likely equally susceptible to the affects of vaccination with PH. Since adrenergic system mediated vasorelaxation is thought to be predominantly through the beta adrenergic and alpha-2 adrenergic receptors disruption of these systems would result in disruption of the ability of the vascular system to vasodilate in response to local or systemic conditions.

The evaluation of two currently used vaccines suggests that there is disruption of the beta adrenergic receptor mechanism after vaccination. Experiemental review of these vaccines is hindered by the fact that they were not evaluated in the target species. However, in view of the similarity of the effect of parenteral exposure to PH in rats and sheep, it is reasonable to expect that there is beta adrenergic receptor disruption in cattle similar to that in the rat.

Micotil 300® has been demonstrated to have beta adrenergic activity at the vascular smooth muscle associated beta adrenergic receptor. Given the physiological significance of the beta adrenergic receptor to vascular physiology and the positive results of the use of terbutaline (a beta-2, selective agonist) in the treatment of endotoxin induced lung injury, the beta adrenergic activity of Micotil 300® suggests that this feature of this macrolide antibiotic serves as a positive adjunct in the treatment of BRDC. Further the in-vitro levels of this drug used are comparable to the tissue concentrations in lung tissue as described by the manufacturer.

The results presented above are limited in their application for several reasons. First, these studies were completed in a non target species. The use of the rat aorta is not anatomically identical to the pulmonary vasculature of the ruminant. Secondly, these studies have been completed on ex-vivo tissue. A model that examines tissue in-vitro fails to recognize and address the dynamic compensation of the vascular system in general and the pulmonary vasculature specifically. Specific compensation in the organism for vascular associated adrenergic dysfunction may render insignificant the findings described above. However there is some support for the biologic significance of the vascular associated adrenergic receptor system in the form of the positive effect of the beta agonist, terbutaline and clinical observations associated with Micotil 300®. Third, it is scientifically reasonable to suggest that the pulmonary vascular associated beta adrenergic receptor is intimately involved with the development of pneumonic pasteurellosis. It is unlikely that this is the initiating factor or even the primary event in the development of the clinical disease. There are very likely a number of events that occur before the disruption of the beta adrenergic receptors. There is at least one event preceding the disruption of beta receptor mechanism disruption, in the pathogenesis of pneumonic pasteurellosis, this is likely the elaboration of inflammatory cytokines. Finally, the intact pulmonary system is not without redundant and multiple compensatory mechanisms that may be able to ameliorate or even eliminate the consequences of BARC

disruption. These protective mechanisms cannot be reproduced in their complexity in the ex-vivo / in-vitro system.

Relevant future studies must include the following. First, the evaluation of the pathophysiology of the PH mediated disruption of beta adrenergic receptors to the bovine pulmonary vascular system. This may be best accomplished by the assessment of pulmonary vascular functioning by use of a Swan-Ganz catheter in the target species. Further, the clinical significance of vascular dysfunction must be evaluated with respect to the in-vivo health and morbidity of the target species.

Secondly the evaluation of the events preceding the vascular associated beta adrenergic mechanism disruption must also be evaluated. The significance of the beta receptor of the pulmonary alveolar macrophage with respect to the elaboration of cytokines is very likely, in my opinion, to be an essential ingredient as to whether or not an individual animal either develops or doesn't develop pneumonic pasteurellosis. It is clear that the immunopharmacology of stress is also integral to this process and must be weighed in the assessment of these events.

Third, the evaluation of the beta receptors involved with the neutrophil per se, the endothelial mediated thrombolic events, the extravasation of intravascular fluid and consequences of disruption of these beta adrenergic receptors must be investigated. The nature of the disruption of the neutrophil based beta adrenergic receptor associated mechanisms may be accomplished by use of the Microphysiometer® (Molecular Devices, Sunnyvale California). Other techniques may be available to evaluate the other beta adrenergic associated mechanisms.

Fourth, examination of the activities of the neutrophil in the development of oxygen free radicals and other chemical species in the development of pneumonic pasteurellosis may suggest ways of treatment of the consequences of pneumonic pasteurellosis.

Finally the evaluation of the affect of PH on vascular associated beta adrenergic receptor mechanisms in the target species under field conditions may allow the correlation of clinical, gross and histopathological signs of the disease with the status and level of the adrenergic system functional pathology at the molecular level. This is likely to make the current research relevant to the real world situation.

Based on these results and those of previous colleagues it is becoming more obvious that the adrenergic system is intimately involved in the etiology and pathogenesis of the disease pneumonic pasteurellosis in cattle and that this disease has profound vascular effects which have previously been under-appreciated until now. Use of this information and manipulation of the vascular parameters discussed in this dissertation may lead to insights for the reduction of a significant bovine disease and the attending economic burden placed on the cattle producers. Hopefully the information presented in this dissertation is one small step towards the resolution of this devastating problem.

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VI. VITAE

Dr. Ernest Rogers was born in Montreal, Quebec, Canada. After graduation from high school he accepted a technician's position in Physiological Psychology at McGill University and worked in the areas of Learning Psychology and the Neurophysiology of Addictive Behaviors. Dr. Rogers then moved to the University of Toronto where he continued his technical career in the study of epileptiform activity (Kindling) of the limbic system. After a move to Vancouver, British Colombia, he took on the position of Veterinary Technician. In May 1979, Dr. Rogers was awarded the diploma, Laboratory Animal Technician from the Canadian Association of Laboratory Animal Science.

In September 1979, he entered the University of Guelph. Dr. Rogers graduated with a Bachelor of Arts (General, Major-Psychology) and a Bachelor of Science (Specialized Honors Biology, Minor Biomedical Sciences) from the University of Guelph in 1985. In August of 1987 Dr. Rogers then entered the professional program of veterinary medicine at Tuskegee University, School of Veterinary Medicine and graduated in May 1991.

Immediately after graduation Dr. Rogers worked in anesthesia at both Tuskegee University and Auburn University. In April 1993 he came to Virginia Tech. to complete graduate studies in immunopharmacology and immunotoxicology under Drs Peter Eyre and Hugo Veit. During his stay at Virginia Tech he has had the opportunity to continue his interest in clinical veterinary behavior by consulting and writing in this area.

Dr. Rogers hopes to advance his career in pharmacology, toxicology and veterinary medicine. He will return to his other passions of SCUBA diving, photography, skiing and flying. He currently lives with his canine companions, Tucker, CoCo, Punk and one feline companion, Tutoro. Unfortunately, their companions Boo and Kodiak have died.