THE ANTIENDOTOXIN EFFECTS OF POLYMYXIN B IN A FELINE MODEL OF ENDOTOXEMIA

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by

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The undersigned, appointed by the dean of the Graduate School, have examined the thesis entitled

THE ANTIENDOTOXIN EFFECTS OF POLYMYXIN B IN A FELINE MODEL OF ENDOTOXEMIA

presented by Claire R. Sharp,

a candidate for the degree of master of science,

and hereby certify that, in their opinion, it is worthy of acceptance.

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Academic Abstract

Introduction: Sepsis, defined as the systemic inflammatory response to infection, is a major cause of morbidity and mortality in veterinary and human patients.[1-5] While the epidemiology of feline sepsis is poorly characterized, report mortality ranges from 22-79%.[6-11] During Gram negative bacterial sepsis, endotoxin or lipopolysaccharide (LPS), the glycolipid component of the cell wall of Gram negative bacteria, is released into circulation (endotoxemia) resulting in a systemic inflammatory response that can lead to multiple organ dysfunction, and death.[12, 13] Directed, effective therapies for feline sepsis are needed to reduce the high morbidity and mortality associated with this disease.

Given that Gram negative endotoxin is perhaps the most powerful stimulus of the sepsis cascade, endotoxin is a logical target for therapeutic intervention in sepsis.[14] A variety of anti-endotoxin strategies have been evaluated for the treatment of endotoxemia and sepsis.[15] Such therapies can be categorized mechanistically; including reducing endotoxin release during bacterial killing with specific antibiotics, increasing clearance of endotoxin from plasma, and antagonizing or inhibiting the effects of endotoxin at cell surface receptors.[13, 15] Polymyxin B (PMB) is one such promising anti-endotoxin therapy.

Polymyxin B is a cyclic cationic polypeptide antibiotic that binds to the lipid A subunit of endotoxin molecules with high affinity, preventing the interaction of endotoxin with humoral and cellular receptors and the resultant activation of inflammatory pathways.[16, 17] The anti-endotoxic effects of PMB are well established both *ex vivo* and *in vivo* in many species,[18-24] including a high-dose lethal endotoxemia model in cats.[25] Based on these studies, treatment with PMB may be beneficial in cats with naturally

developing Gram negative sepsis, although evaluation of the anti-endotoxic effects of PMB in a clinically applicable experimental model of feline sepsis was thought to be a logical first step prior to clinical use given historical concerns with drug toxicity.

In this study we investigated the anti-endotoxin effects of PMB using a previously characterized, low-dose endotoxin infusion model of Gram negative feline sepsis in minimally instrumented, conscious cats.[26] We hypothesized that PMB would ameliorate endotoxin-induced systemic inflammation and hemodynamic derangement with minimal adverse effects.

Materials and Methods: We investigated the anti-endotoxin effects of PMB in a blinded, placebo controlled fashion, both *ex vivo* in a feline whole blood culture system and i*n vivo*, using a low-dose endotoxin infusion in cats (2ug/kg/hr IV x 4 hours). Serial measures of systemic inflammation, and hemodynamic stability, were compared between groups.

Specifically, rectal temperature, heart rate, and Doppler systolic arterial blood pressure were evaluated at baseline and then every 30 minutes for 6 hours after initiation of LPS infusion. Blood was collected at baseline and serially for complete blood counts, and plasma tumor necrosis factor (TNF) activity. Cats were also evaluated for signs of toxicity, including neurotoxicosis (serial neurologic examination), anaphylaxis, gastrointestinal disturbance, respiratory depression (respiratory rate and character) and nephrotoxicosis (urinalysis).

Results: *Ex vivo*, PMB significantly decreased LPS-induced TNF production from whole blood. *In vivo*, endotoxin infusion resulted in the development of fever, hypotension, leucopenia and increased TNF activity. Polymyxin B (1mg/kg over 30 minutes) treatment

decreased peak plasma TNF activity ($p<0.001$) and increased white blood cell count ($p=0.019$), with no adverse effects.

Conclusions: Polymyxin B administration resulted in decreased peak plasma TNF activity and increased white blood cell count in this feline model of endotoxemia, with no adverse effects. Given the apparent safety and anti-endotoxin effects of PMB in this endotoxemia model, a carefully designed, randomized, blinded, placebo controlled clinical trial evaluating the use of PMB in naturally occurring Gram negative feline sepsis should be considered.

Chapter 1: Anti-endotoxin Strategies for the Prevention and Treatment of Gram Negative Bacterial Sepsis

Sepsis, defined as the systemic inflammatory response to infection, is a major cause of morbidity and mortality in veterinary and human patients.[1-5] Gram negative bacterial infections are a common cause of sepsis. During Gram negative sepsis, endotoxin or lipopolysaccharide (LPS), the glycolipid component of the cell wall of Gram negative bacteria, is released into circulation resulting in a systemic inflammatory response that can lead to multiple organ dysfunction, and death.[12, 13] Endotoxemia is defined as the presence of endotoxin in circulation, or more specifically "the clinical syndrome when endotoxin is presumed to be present".[27] There is likely considerable overlap between the syndromes of sepsis and endotoxemia as Gram negative bacterial endotoxin is a potent stimulator of the innate immune response that induces systemic inflammation and the pathologic sequelae of sepsis.[12, 13, 15]

Sepsis and its sequelae are the leading cause of death in critically ill human patients, thus generating extensive preclinical and clinical research interest in attempts to better understand and therapeutically modulate this perturbation of the inflammatory response.[28, 29] However, despite the devastating nature of sepsis in both human patients and our veterinary species, and advances in our understanding of this disease, mortality remains high (28.6-38.4% in people;[30] 33-64% in dogs;[31-33] 22-79% in cats[6-11]). Current therapy for sepsis still relies largely on intensive supportive care and attempts at eliminating the underlying microbial etiology, with little success in clinical trials

targeting inhibition of specific inflammatory mediators, so called mediator-directed therapy for sepsis.[3, 28, 30, 34] Given the vital role that the innate immune response plays in the development of sepsis, it is not surprising that various components of the innate immune response to endotoxin have been evaluated as putative therapeutic targets.[15, 35] In the 'early days' of mediator-directed therapy, the concept of early removal of endotoxin from the circulation of patients with Gram negative sepsis, in order to attenuate the overactive immune response and excessive pro-inflammatory mediator release, was viewed as not only logical but perhaps the most ideal therapeutic strategy for sepsis.[15] Antiendotoxin strategies have consistently demonstrated survival advantage in experimental, animal models of sepsis; however, thus far this has not translated into the clinical realm.[15]

This introductory chapter will review the preclinical and clinical literature in regards to antiendotoxin strategies by sequentially evaluating potential points of intervention at each stage of LPS signaling through the innate immune response.

Endotoxin / Lipopolysaccharide

Lipopolysaccharide is a logical therapeutic target in Gram negative sepsis in that it is perhaps the most powerful and broad-spectrum stimulus to the sepsis cascade, acting as both an initiator and propagator of sepsis.[14] Endotoxin biosynthesis is unique to Gram negative bacteria and LPS has a remarkably conserved structure across Gram negative bacterial species.[12, 15]

a. Structure

Lipopolysaccharide consists of three interconnected structural components; lipid A, a core oligosaccharide and an O-specific oligosaccharide side chain.[14, 36, 37] The lipid A component is responsible for the endotoxic properties of the molecule, and is the most highly conserved region (although not identical) among Gram negative bacterial species.[15] The core oligosaccharide consists of both an inner core of heptose, KDO [3-deoxy-D-manno-octulosonic acid] residues and hexose molecules; and an outer core of more variable sequences of hexose molecules.[36] The O-specific oligosaccharide side chain is exposed on the outer surface of the bacteria and is the immunodominant region of the LPS structure.[38, 39]

b. The role of endotoxin in the early events in Gram negative sepsis

The interaction of endotoxin with components of the innate immune system occurs initially extracellularly, before engaging with cell surface toll-like receptors (TLRs) and initiating an intracellular signaling cascade that ultimately results in the upregulation of nuclear transcription factors and hence gene transcription, particularly for proinflammatory mediators.[15, 40] In brief, during Gram negative sepsis, LPS (in fragments of bacterial cell walls and even on whole bacteria) in circulation binds the acute phase protein, LPS binding protein (LPSBP or LBP). This complexing of LPS and LBP facilitates the delivery of LPS to the glycosylphosphatidylinositol[GPI]-anchored cell surface receptor membrane cluster of differentiation-14 (mCD14) on cells of the immune system.[41] Although alternative mechanisms for LPS transfer to mCD14 may exist, this process occurs much less efficiently in the absence of LBP. While CD14 is considered a pattern recognition receptor (PRR) of the innate immune response, it lacks a membrane-

spanning domain, and thus is unable to initiate intracellular signaling. Signal transduction thus requires a transmembrane receptor; toll-like receptor 4 (TLR4), in the case of LPS.[42] Binding of LPS to CD14 activates TLR4, and its accessory protein lymphocyte antigen 96 (known as MD2), initiating several intracellular signal cascades that ultimately leads to activation of nuclear transcription factors, including nuclear factor kappa B (NF-kB) and activator protein 1 (AP-1). These transcription factors subsequently coordinate the induction of many genes encoding pro-inflammatory mediators.[43]

Gene products of these transcription factors include cytokines [e.g. tumor necrosis factor-alpha (TNF-α), interleukin-1-beta (IL-1β), interleukin-6 (IL-6)], chemokines (e.g. CXCL-8 or IL-8), nitric oxide, products of the complement and arachidonic acid cascades and reactive oxygen species.[40, 44] These pro-inflammatory mediators drive the development of inflammation, which locally is manifested as increased blood flow, activation of coagulation, increased local metabolism, increased vascular permeability and cellular infiltration.[4] These 'cardinal signs of inflammation' represent potentially beneficial mechanisms to control infectious agents, but if there is an excessive pro-inflammatory response or inadequate anti-inflammatory response to control the inflammation, sepsis can result.[2] Clinically, sepsis manifests as dysregulation of body temperature (fever or hypothermia), heart rate (tachycardia or bradycardia), respiratory rate (tachypnea) and leukocyte count (leukocytosis or leucopenia).[2, 45] Pathophysiologic consequences of sepsis include disordered coagulation, cardiovascular alterations including septic shock, metabolic derangement, acute lung injury and other multiple organ dysfunction.[4, 9, 44, 46]

While endotoxin is a product of Gram negative bacteria, different pathogen associated molecular patterns (PAMPs) are found on other microbes that engage the innate immune response (e.g. lipotechoic acid from Gram positive bacteria[47]) resulting in systemic inflammation that can cause gastrointestinal barrier dysfunction and the translocation of Gram negative bacteria or endotoxin into systemic circulation.[42] As such it is theorized that endotoxemia may be a common feature of sepsis from any etiology. Consistent with this theory, clinical studies indicate that endotoxin is found in the systemic circulation of the majority of human patients with septic shock and this endotoxemia is largely independent of the nature of the infecting microorganism.[15, 48-50]

c. Experimental animal models of endotoxemia

Animal models are used extensively in sepsis research, the reason being that rigorously evaluating the safety and efficacy of novel therapies is potentially risky in clinical patients, and often requires serial invasive procedures which may be deleterious in these already critically ill and unstable patients.[51] An understanding of the features and limitations of these models is vital to understanding the extensive preclinical literature regarding the potential therapeutic benefit of antiendotoxin strategies and other therapeutic targets for the treatment of Gram negative sepsis.[52, 53]

Rodent models are perhaps the most commonly used animal model of sepsis.[54] Various techniques can be used to produce an experimental sepsis-like syndrome; including intravenous infusion of endotoxin alone, intravenous infusion of whole bacteria (particularly *Eschericia coli*) and models of abdominal sepsis.[51, 52] In regards to endotoxemia models, various concentrations / doses of LPS from various bacterial species (most commonly *E.coli* and *Salmonella* spp.) have been used to induce

everything from lethal septic shock to milder, almost self-limiting clinical signs of sepsis.[55, 56] Abdominal sepsis is generally created by cecal ligation and puncture (CLP) or colon ascendans stent peritonitis (CSAP), both of which produce mixed bacterial sepsis from septic peritonitis.

Rodent species tend to be popular for modeling sepsis because they are small and relatively inexpensive, allowing larger numbers of animals to be used.[55, 57] In addition, the genetic characteristics of inbred strains of mice are known, knockout and transgenic mice are available, and a wide array of reagents for immunologic studies are produced commercially.[54] Disadvantages of rodent models include their small size, precluding easy monitoring of cardiovascular parameters and repeated blood sampling, and marked species differences compared to humans in susceptibility to endotoxemia, cell signaling, TLRs, inflammatory mediators etc.[51] These limitations of rodent models may explain, at least in part, the failure of translation of preclinical success into the clinical realm.[52, 55]

Large animal models are advantageous in that they are more likely to mimic the heterogenous genetic population and reproduce the gradual pathophysiologic changes seen in humans with sepsis.[58, 59] Large animal models of sepsis have included a variety of species including dogs, cats, pigs, sheep, and rabbits. [21, 25, 53, 58, 60, 61] Nonhuman primates, including baboons, cynomolgus macaques, and rhesus macaques, have also been used in some sepsis studies to closely replicate the human inflammatory response.[59] However, disadvantages of using nonhuman primates, such as ethical concerns, substantial cost, and the potential for transmission of zoonotic disease, have limited their use to preclinical studies and investigations in which a small sample size is sufficient.[57, 60]

Large animal sepsis models have greatly expanded our collective knowledge of the pathophysiology of sepsis in both human and veterinary patients. While none of the large or small animal models reproduce all of the physiologic and immunologic consequences of human sepsis, valuable information can be derived from each model when data are interpreted with regard for the limitations of that model. In fact, full evaluation of promising treatments for sepsis may require assessment using a series of animal models of increasing complexity.[54]

Systematic discussion of specific antiendotoxin strategies:

a. Reduced LPS production and release:

Lipid A and 3-deoxy-D-manno-2-octulosonate (KDO) synthesis inhibitors

The biosynthetic pathways for lipid A, and the KDO molecules of the inner core of LPS, are attractive targets for inhibition since the enzymes in these pathways are conserved in most Gram negative bacteria and are generally essential (with a few exceptions) for bacterial growth and viabiblity.[36, 62, 63] In recent years our understanding of the structure and function of various Gram negative outer membrane biosynthetic enzymes has greatly advanced, facilitating the development of specific inhibitors. Compounds that inhibit these pathways, not only have significant direct antibacterial activity but also greatly enhance the susceptibility of Gram negative bacteria to serum-mediated killing through phagocytosis and complement mediated lysis.[64] Development of antimicrobials which inhibit lipid A and KDO synthesis is ongoing. Preliminary *in vitro* results are promising, however to the knowledge of the authors these compounds have not yet been evaluated *in vivo* either in experimental animal models of sepsis or naturally

developing disease. Future research in this field may allow the development of new classes of antibiotics that can be implemented in clinical trials.[65, 66]

Antibiotics that reduce the release of endotoxin associated with bacterial killing

Appropriate selection of antimicrobial agents and early institution of antimicrobial therapy is vital for the successful treatment of sepsis and minimization of mortality,[67-69] but the administration of antimicrobial agents is not without consequence. When treating patients with Gram negative sepsis, clinicians are faced with the therapeutic dilemma that while antibiotics neutralize and kill the bacteria responsible for the inciting infection, they generally do not neutralize the endotoxin released by bacterial lysis and, in fact, antibiotics may markedly (as much as 20-fold the pre-antibiotic concentrations) increase the bioavailability of bacterial endotoxin.[70] Increased concentrations of pro-inflammatory cytokines (TNF-α, IL-1β, IL-6 etc.) have also been shown to occur following antibiotic administration both *in vitro* and *in vivo*.[71, 72]

The first observation that antibiotic administration could have deleterious effects on patients was made independently by Jarisch and Herxheimer; these effects are now referred to as the Jarisch-Herxheimer reaction (JHR).[73] This syndrome manifests in human patients with various infectious diseases, such as syphilis, leptospirosis,[74] and tick-borne relapsing fever.[75, 76] The JHR is characterized by transient pyrexia, hypotension, and rigors as early as 2 hours after the first dose of antibiotic. While the exact mechanism of this reaction is unknown, there is evidence both for[76-79] and against[70, 80, 81] the role of endotoxin as the biological mediator of this syndrome.[82]

There is accumulating evidence that different antibiotics result in differential LPS release during bacterial lysis. While the full clinical significance of this is not yet known,

the potential for differential LPS release should be considered when selecting both an antibiotic drug, and dose, for the treatment of Gram negative bacterial sepsis.[83-85] Essentially, antimicrobials that result in rapid bacterial lysis minimize LPS release; these include fluoroquinolones, glycopeptides, some of the aminoglycosides, carbapenems, and β-lactam antibiotics which bind preferentially to penicillin-binding-protein-1 (PBP-1), such as cefsulodine and cephalonide. In contrast, using β-lactam antibiotics that bind preferentially to PBP3, such as piperacillin, and ceftazidime, especially at lower doses, result in slower bacterial killing and more LPS release.[85] Further work in a clinical setting is indicated to determine if careful selection of antimicrobial agents may limit the adverse consequences of LPS release during antibiotic therapy and positively impact survival in Gram negative sepsis in both human and veterinary patients.[15]

b. Accelerated LPS clearance:

Phospholipid LPS binders

Lipopolysaccharide complexed to endogenous plasma proteins such as high density lipoprotein (HDL), and to a lesser extent low density lipoprotein (LDL), is metabolically and immunologically inactivated and cleared from the circulation as part of the process of endotoxin detoxification.[13, 86] High density lipoproteins also act through other mechanisms to decrease the inflammatory response to endotoxin including downregulation of pro-inflammatory cytokines and cell-surface adhesion molecules, and upregulation of endothelial nitric oxide synthase (eNOS).[86] Critically ill human patients, including patients with sepsis, often have low concentrations of circulating HDLs and blunted endotoxin detoxification, which is associated with a significantly worse outcome.[87, 88] Similarly, dogs with parvoviral enteritis have decreased concentrations of

circulating HDLs which may impair their ability to clear endotoxin and promote a sustained systemic inflammatory response to endotoxin. [89] To date, HDL concentrations have not been evaluated in a wider population of critically ill dogs.[89]

A variety of animal models have been used to investigate the antiendotoxin properties of exogenously administered phospholipids and lipoproteins.[90] Transgenic mice expressing excessive concentrations of plasma HDLs or wild-type mice treated with exogenous HDLs are protected from endotoxin challenge.[91, 92] Similarly, HDL administration attenuates the deleterious effects of endotoxin in humans, rabbits and pigs, with experimental Gram negative endotoxemia.[93-95]

In veterinary medicine, Moore and colleagues recently evaluated the effects of pretreatment by rapid IV infusion of a phospholipid emulsion (PLE; 100mg/kg IV over 30 minutes) on experimental *E.coli* endotoxemia in horses.[96] Horses receiving the PLE had reduced clinical signs of endotoxemia (lower heart rate and temperature), a reduced leukopenic response and reduced serum TNF-α concentrations, with negligible side effects, when compared to saline controls. The authors concluded that further *in vitro* and *in vivo* studies are warranted to characterize the beneficial effects of PLE in endotoxemic horses.[96] To the knowledge of the authors, PLE therapy for endotoxemia or sepsis has not been evaluated in dogs and cats.

Extracorporeal hemofiltration

Plasmapheresis refers to the removal, treatment (to remove chosen components) and subsequent return of plasma from circulation. In the setting of endotoxemia, plasmapheresis can be used to remove endotoxin and/or inflammatory mediators from circulation. Hemofiltration or hemoperfusion using a PMB-immobilized fiber column,

facilitating removal of plasma endotoxin by binding to lipid A, is effective at reducing endotoxemia in rodent models of sepsis,[19] and naturally developing sepsis in people.[97, 98] To the knowledge of the authors, hemofiltration has not been evaluated in dogs or cats with endotoxemia or sepsis.

Anti-LPS antibodies

Administration of anti-LPS antibodies constitutes a form of passive immunotherapy, with the goal of removing circulating LPS to prevent activation of the innate immune response.[99, 100]

The first clinical trials of anti-LPS antibodies in human medicine used polyclonal sera obtained from healthy volunteers that had been vaccinated with heat-killed *E.coli* 0111-mutant J5. A study by Ziegler et al. documented improvement mortality in Gram negative sepsis and septic shock;[101] however subsequent studies were unable to replicate a beneficial response and, as a result, the use of polyclonal serum went out of favor.

Monoclonal antibodies against lipid A were subsequently developed with the hope of overcoming the problems with serum-based, polyclonal antibody preparations. Multiple monoclonal anti-lipid A antibody preparations have been evaluated in human sepsis clinical trials with variable success, however none are currently commercially available for clinical use.[102-104] Interestingly, increased mortality occurred when one such human IgM monoclonal antibody (HA-1A) was administered to dogs in a model of Gram-negative septic shock.[105]

Hyperimmune plasma refers to plasma harvested from individuals that have been previously immunized with, or naturally exposed to, specific microbial antigens such that

it contains high concentrations of particular antimicrobial antibodies. The rationale for the use of hyperimmune plasma is that anti-lipid A antibodies will bind LPS, preventing interaction with and activation of the mononuclear phagocyte system and subsequent induction of the pro-inflammatory response. The use of hyperimmune plasma has been evaluated by multiple investigators in equine endotoxemia with conflicting results, ranging from no improvement in consequences of endotoxemia,[106, 107] to reduced clinical signs of endotoxemia and reduced mortality, [108109] to worsening of clinical signs and inflammatory mediator concentrations.[110] Similarly the results of studies evaluating polyvalent equine-origin anti-endotoxin antiserum (SEPTI-Serum®, IMMVAC, Columbia, MO) in dogs with parvoviral gastroenteritis have yielded conflicting results, with some studies suggesting an improvement in mortality,[111] and others documenting increased mortality.[112] Despite a lack of conclusive evidence of efficacy in horses or dogs, these products are used clinically by veterinarians.

Immunoglobulin (IVIG) supplementation

Intravenous immunoglobulin (IVIG) is an immunoglobulin preparation from the pooled plasma of human donors. It contains a broad repertoire of antibodies but is fractionated to contain predominantly IgG (at least 90%), with a normal subclass distribution; as well as small quantities of IgA, IgM, IgD and IgE, and trace amounts of soluble CD4, CD8 and human leukocyte antigen (HLA) molecules, cytokines and intact Fc (fragment, crystallizable) molecules. Following infusion in human patients, the half-life averages 21to 25 days, with ~55% distributing extravascularly. The half life in dogs is relatively short at 7 to 9 days.[113]

The mechanisms of IVIG are complex and reflect the numerous functions of circulating immunoglobulins including Fc receptor blockade, attenuation of complement and cytokine mediated damage, and modulation of B- and T-lymphocyte activation and effector functions. In experimental studies it has been shown that polyvalent Igs can provide broad cross-reactivity against a number of potential antigens (both known and unknown). Proposed mechanisms of action of polyvalent immunoglobulins in sepsis include their ability to improve opsonization of infecting microbes facilitating phagocytosis and killing, prevention of nonspecific complement activation, protection against antibiotic induced liberation of LPS; and neutralization of LPS and a wide variety of other superantigens.

There is ongoing debate about the efficacy of polyvalent immunoglobulins in preparations such as IVIG, as adjunctive therapy for sepsis or septic shock. A recent meta-analysis evaluating the use of IVIG in sepsis included 27 trials with a total of 2,202 human patients .[114] This review concluded that polyvalent Igs exert a significant positive effect on survival in sepsis and septic shock (relative risk of death of 0.79) with a strong trend in favor of an Ig preparation enriched with IgA and IgM (IgGAM; reducing mortality by 34% in adults and 50% in neonates) compared with preparations containing only IgG.[114] This is likely since the IgGAM preparations contain higher titers of antibodies to a greater number of bacterial pathogens, and supply more opsonins.[115, 116]

The use of human IVIG has not been reported in septic veterinary patients.

c. LPS antagonism:

Synthetic and naturally occurring antagonist, and partial agonist-antagonists, of the lipid A portion of LPS molecules have been developed. Lipid A antagonists including

polymyxin B, surfactin C, and lipid X have been evaluated in various experimental and clinical settings. [38, 117]

Polymyxin B

Polymyxins are a group of polypeptide antibiotics characterized by a heptapeptide ring, a high content of diaminobytryric acid, and a side chain ending in fatty acid. Polymyxin B is a cyclic cationic polypeptide antibiotic that binds to the lipid A subunit of endotoxin molecules with high affinity, preventing the interaction of endotoxin with humoral and cellular receptors and the resultant activation of inflammatory pathways.[16] PMB has been shown to inhibit activation of nuclear factor kappa B (NF-κB), one of the primary cytokine transcription regulators during Gram negative sepsis.[118] It is through these mechanisms that PMB prevents the production of cytokines like TNF and hence the development of systemic inflammation.[24]

The anti-endotoxic effects of PMB are well established in many species. Polymyxin B has been shown to protect mice against lethal endotoxin toxin challenge.[20] Polymyxin B also moderates the deleterious effects of established, overwhelming gram negative sepsis, such as hypotension and acidosis, induced by intraperitoneal injection of *E.coli* in rabbits.[20] Intravenous administration of PMB (1 mg/kg = 6000 U/kg IV every 8 hours) has been proven to be an efficacious, affordable and safe means of inhibiting endotoxin-induced inflammation in horses experimentally,[18, 22, 24] and is routinely used in this species clinically.[23] Similar anti-endotoxic effects of PMB have been documented both experimentally and clinically in dogs.[21, 119]

In a canine model of endotoxemia, incubation of LPS with PMB prior to injection, resulted in a significant reduction in mortality from 86% to 14% in the PMB treated

group.[21] Polymyxin B has also been used successfully and safely in naturally developing canine sepsis secondary to parvovirus gastroenteritis, resulting in a significant reduction in plasma TNF concentrations compared to control; a particularly important outcome measure since plasma TNF concentration is directly associated with sepsis mortality in dogs. [119, 120]

Until this time, only one study has evaluated PMB in feline endotoxemia. In this study performed over 25 years ago, PMB (5 mg/kg bolus + 5 mg/kg continuous rate infusion over 30 min, IV) was administered in a high dose endotoxin model of sepsis in cats. In this model, PMB treatment dramatically improved survival rates (from 12.5 to 100%) and prevented the endotoxin induced pulmonary hypertension, systemic hypotension and reduced cardiac output.[25] Although compelling, the severe nature of the endotoxin insult, extreme instrumentation, and use of anesthesia limits the clinical application of these data. Additionally, the total dose of PMB of 10 mg/kg has been documented to result in respiratory arrest in cats, but the cats in this study were mechanically ventilated, so respiratory effects could not be evaluated. Nevertheless, the dramatic improvement in survival with PMB treatment cannot be ignored.

Concerns about the toxic effects of PMB have limited its clinical use, both as an antibiotic and for its anti-endotoxin effects, in non-equine species. The toxicity of PMB results from its ability to bind to phospholipid membranes with the potential to disrupt cellular function in multiple organ systems.[121] Nephrotoxicosis, neurotoxicosis, respiratory arrest, profound cardiovascular dysfunction and histamine release have been reported as potential side effects. Adverse effects are however considered to be dose dependent and resolve with cessation of treatment. [17, 121-123] In cats, hypoventilation

secondary to neuromuscular blockade occurs at doses of 3 mg/kg (18,000 U/kg) and respiratory arrest occurs at doses of 5 mg/kg (30,000 U/kg).[124] In other species, substantially lower doses of PMB (1 to 2 mg/kg) have been shown to maintain considerable anti-endotoxic effects while avoiding adverse consequences.[23]

Surfactin C

Surfactin isomers, classified as A, B and C based on differences in their amino acid sequences, are macrolide lipopeptides produced by various strains of *Bacillus subtilis*.[125] Like the polymyxins, the surfactins contain a heptapeptide ring, in this case linked via a lactone bond to a β-hydroxy-fatty acid side chain.[126, 127] Surfactins were so named for their powerful surface tension-lowering activity (i.e. they act as biosurfactants). Additional properties of surfactins include antitumor,[128] anti- inflammatory,[129] antibacterial,[130, 131] and antiviral activity.[132] Surfactin C has been reported to inhibit the transcription of inducible NOS mRNA and the production of NO induced by LPS in murine macrophage cells.[133] To the knowledge of the authors surfactin, C has not been evaluated in dogs, cats, horses or people with endotoxemia or sepsis.

Lipid X

Lipid X, a synthetic antagonist of lipid A, has been evaluated in a canine model of Gram negative sepsis. At the low dose evaluated no difference between lipid X and placebo treated dogs was evident; at the higher dose lipid X was unexpectedly found to decrease survival.[134]

d. LPS/CD14/TLR-4 interaction inhibition:

Anti-CD14 antibodies

The vital role of CD14 in directing LPS responses was first demonstrated *in vivo* in mice. CD14 knockout mice are insensitive to doses of LPS that produce 100% mortality in CD14 positive litter mates, and significantly less susceptible to Gram negative infections.[135] In a primate model of endotoxin-induced septic shock, treatment with anti-CD14 antibodies also resulted in reduced mortality.[15] However, in a rabbit model of *E.coli* pneumonia and sepsis, blockade of CD14 by anti-CD14 antibodies led to greater bacterial dissemination and increased mortality, if appropriate antibiotics were not administered concurrently, due to dampening of the patients protective responses.[35, 61] Monoclonal anti-CD14 antibodies (IC14), evaluated in a phase Ib/II clinical trial in human patients with severe sepsis, are well tolerated but their effect on outcome remains to be determined.[15] Anti-CD14 antibodies have not been evaluated in veterinary medicine.

TLR-4 receptor blockade

Eritoran (E5564), a specific TLR4 antagonist, has recently been evaluated in a series of human clinical trials.[15] A double-blind, placebo-controlled trial demonstrated that Eritoran blocked some of the toxic effects of endotoxin infusion in healthy human volunteers, ameliorating the fever, tachycardia, leukocytosis and pro-inflammatory cytokine production.[136] Improved mortality was demonstrated in a phase II trial in septic humans, and phase III clinical trials are currently underway.

Conclusions

"Animals that cannot sense endotoxin may die if they are infected by Gram negative bacteria. Animals that sense endotoxin and respond too vigorously, as in the cases of sepsis and septic shock, may also die, victims of their own inflammatory

reactions."[13] The innate immune response to endotoxin is a highly complex and dynamic process involving LPS interaction with cell surface PRRs (notably TLR4) and intracellular signaling pathways that culminate in upregulation of gene transcription for pro-inflammatory mediators, whose actions generate the clinical manifestations of local infection and sepsis. While the history of therapeutic interventions in clinical trials for sepsis has been referred to as the "graveyard for pharmaceutical companies", ongoing research, particularly regarding the modulation of the innate immune response, provides hope for new approaches that will be therapeutically effective in the treatment of sepsis.[15, 28, 29]

Chapter 2 – The antiendotoxin effects of polymyxin B in a feline model of endotoxemia

Sepsis, defined as the systemic inflammatory response to infection, is a serious problem in feline patients causing substantial morbidity and mortality.[9, 137] In cats, Gram negative bacterial infections are a common cause of sepsis.[9, 138-142] During Gram negative sepsis, endotoxin or lipopolysaccharide (LPS), the glycolipid component of the cell wall of Gram negative bacteria, is released into circulation resulting in a systemic inflammatory response that can lead to multiple organ dysfunction, and death.[12, 13] Despite the potentially devastating nature of sepsis in cats, little research has focused on treatment and current therapy relies on control of the source of infection, appropriate antimicrobial coverage and aggressive supportive care. Specific directed therapies for sepsis in cats have not been developed.

Polymyxin B (PMB) is a cyclic cationic polypeptide antibiotic that binds to the lipid A subunit of endotoxin molecules with high affinity, preventing the interaction of endotoxin with humoral and cellular receptors and the resultant activation of inflammatory pathways.[16, 17] Polymyxin B, by neutralizing LPS, inhibits activation of nuclear factor kappa B (NF-κB),[118] one of the primary cytokine transcription regulators during Gram negative sepsis.[143, 144] The antiendotoxic effects of PMB are well established both *ex vivo* and *in vivo* in many species, including experimental models of sepsis in rabbits,[20, 145] rodents,[146] dogs,[21] horses,[18, 23, 24] and humans,[147] as well as naturally

occurring parvoviral gastroenteritis induced sepsis in dogs.[119] Treatment with high dose IV PMB in a high dose feline endotoxemia model has been associated with a dramatic improvement in survival (from 12.5% in the placebo group to 100% in the PMB treated group).[25] Although compelling, the administration of PMB prior to endotoxemia, high total dose of PMB, severe nature of the endotoxin insult, extreme instrumentation, and use of anesthesia in that study limits the clinical application of these data. Nevertheless, the dramatic improvement in survival with PMB treatment cannot be ignored. Based on these studies, treatment with PMB may be beneficial in cats with naturally developing Gram negative sepsis, although evaluation of the antiendotoxic effects of PMB in a clinically applicable experimental model of feline sepsis was thought to be a logical first step prior to clinical use.

PMB is readily available, inexpensive, easily administered and has endotoxin neutralizing effects that long surpass its circulating pharmacological half-life making it an attractive drug for the treatment of Gram negative sepsis.[18, 24] We hypothesized that PMB would ameliorate endotoxin-induced systemic inflammation and hemodynamic derangement with minimal adverse effects. In this study we investigated the anti-endotoxin effects of polymyxin B using a previously characterized, low-dose endotoxin infusion model of Gram negative feline sepsis in minimally instrumented, conscious cats.[26]

Materials and Methods:

Ex vivo preliminary data:

Feline whole blood culture (Cwb) was used to investigate if PMB blunted LPS-induced whole blood TNF production in cats, *ex vivo*. Adult male cats, which belong to a colony

maintained at the University of Missouri, were used for this part of the study, which was approved by the Animal Care and Use Committee at the University of Missouri. All cats were known to be healthy on the basis of a normal physical examination and complete blood count (CBC) and plasma biochemical analysis. Cats were cared for according to the principles outlined in the NIH Guide for the Care and Use of Laboratory Animals. Twelve milliliters of blood was collected from each cat into sodium heparin blood tubes. The cats have previously had vascular access ports (Norfolk Vet Products, Skokie, IL) surgically implanted in their right jugular vein; blood samples were taken from these ports using a three-syringe technique. Vascular access ports are maintained long-term in these cats; their patency is achieved by 'locking' them with heparin after each use. Within one hour of blood collection, the blood samples were mixed to achieve a 1:2 dilution with Roswell Park Memorial Institute **(**RPMI) culture media (RPMI, 200 U Penicillin/ml, 200 ug streptomycin/ml and 200 mM l-glutamine). The Cwb was stimulated with either LPS (1000 ng/mL) or a control solution (phosphate buffered saline [PBS]) with and without PMB (1, 5, 10, 25 ug/mL) in 12 well plates as previously described [148]. Plates were incubated at 37°C with 5% CO_2 for 24 hours before centrifugation (1000 g x 10 min), and collection of the supernatant. Samples were then frozen at -80°C until analysis. A TNF assay was performed on cell culture supernatant.

TNF activity - TNF activity was evaluated in Cwb using a previously described cytotoxicity bioassay.[149-152] Briefly, cells from mouse fibroblast cell (L929) were cultured on 96 well plates. After 12 hours, samples were added to the wells in triplicate. After a 20 hour incubation with MEM (minimum essential medium) plus horse serum and

actinomycin D (Sigma-Aldrich, St. Louis, MO), 3-[4,5-dimethylthiazol-2-yl]-2,5,-di-phenyl tetrazolium bromide (MTT) (Sigma-Aldrich, St. Louis, MO) was added and the cells were incubated for an additional 2.5 hours to allow for formazen crystal formation. The formazen crystals were then solubilized in dimethylformanide and SDS (Sigma-Aldrich, St. Louis, MO). Color development after 1 hour was measured at 630 nm. Feline rTNF (Endogen, Rockford, IL) was used to construct a standard curve to determine the concentration of TNF activity in the test wells. The lower limit of detection for this assay is 0.5 ng/mL.

In Vivo Study

Animals: Following the *ex vivo* pilot study, twelve adult, sexually intact, specific pathogen free (SPF) male cats were purchased (Liberty Research, Waverly, NY) and used in a randomized, blinded, placebo controlled *in vivo* study. The study was approved by the Animal Care and Use Committee at the University of Missouri. Animals were cared for according to the principles outlined in the NIH Guide for the Care and Use of Laboratory Animals. The health of the cats was confirmed based on physical examination and assessment of renal function (i.e. normal plasma creatinine concentration). The cats were one year old with a mean weight of 4.9 kg. The cats were maintained on commercial adult cat food and water *ad libatum*, but fasted each day prior to procedures.

Treatments: On day one, the cats were instrumented with a 4 Fr single lumen jugular catheter (Cook Veterinary Products, Bloomington, IN) and a 22 G cephalic catheter (Abbott, Abbott Park, IL) during brief sedation with a combination of IV medetomidine

(15 mcg/kg Domitor, Pfizer Animal Health, Exton, PA) and butorphanol (0.2 mg/kg Torbugesic, Fort Dodge, Fort Dodge, IA). On day 2, all cats received a 4 hour, 2 mcg/kg/h IV *Escherichia coli* 0127:B8 LPS infusion (Sigma-Aldrich, St.Louis, MO) starting at baseline. Thirty minutes after the initiation of the LPS infusion, cats received a 30 minute IV infusion of either 10 mL 0.9% NaCl (placebo) or 1 mg/kg PMB diluted to 10 mL with 0.9% NaCl (Polymyxin B for Injection, Bedford Laboratories™, Bedford, OH). Cats were randomized to treatment groups using a table of random numbers. Crystalloid fluid (0.9% NaCl) was administered to any cat that developed severe hypotension (Doppler systolic blood pressure [BP] < 60 mmHg) in 10 mL/kg increments to achieve resolution of severe hypotension (BP > 60 mmHg).

Sample collection: Rectal temperature (T), respiratory rate (RR) and character, heart rate (HR), Doppler (Parks Medical Electronics, Las Vegas, NV) systolic arterial blood pressure (BP) and neurologic examination (assessment of mentation, posture and cranial nerve function) were evaluated at baseline and then every 30 minutes for 6 hours after initiation of LPS infusion. Cats were also evaluated for signs of anaphylaxis (including angiedema, urticaria, pruritis, respiratory distress, vomiting and hypotension) and development of gastrointestinal signs (vomiting, diarrhea, salivation). Blood was collected at baseline from the jugular catheter and at predetermined time points thereafter (0.5, 1, 1.5, 2, 3, 4 and 6 hours) into potassium EDTA anticoagulated tubes for complete blood counts (CBC), and lithium heparin tubes (1, 1.5, 3 and 4 hours) for plasma TNF activity. No more than 4 mL/kg of whole blood was drawn in total. For each volume of blood collected, an equal volume of 0.9% saline was administered IV. In addition, urine

was collected via cystocentesis at 6 hours. Plasma and urine supernatant were harvested immediately using a centrifuge (300 Xg for 6 minutes), and banked at -200 °C until analysis.

Assays:

Complete blood count (CBC) – Complete blood counts were performed using a Coulter Counter 880 (Coulter Electronics, Hialeah, FL).

Plasma and urine biochemistry—Urine GGT was measured 6 hours after placebo or PMB treatment. Urinalysis (including dipstick [Multistix®, Bayer, Pittsburgh, PA], urine specific gravity [USG] by refractometry and sediment examination) was also performed on the urine sample collected at 6 hours.

TNF activity – Plasma TNF activity was evaluated at baseline, 1, 1.5, 3 and 4 hours after initiation of LPS infusion using a cytotoxicity bioassay, described above. These time points were chosen based on the expected peak plasma concentration after low dose endotoxin infusion in cats.[26]

Data analysis: Statistical analyses were performed using commercially available software (SigmaStat, Systat Software Inc., Chicago, IL). The Kolmogorov-Smirnov statistical test for normality was used to determine if data were normally distributed. Data that were not normally distributed were transformed using a natural logarithm prior to analysis. Treatments were compared using a repeated measures analysis of variance with post-hoc Tukey multiple comparison procedure. A p-value of < 0.05 was considered statistically significant. Data are expressed as mean± SE unless otherwise noted.

Results:

Ex vivo LPS-induced TNF production from feline Cwb: Whole blood stimulated with LPS produced significantly more TNF activity than control ($p<0.001$) in the absence of PMB (Figure 1). Supernatant TNF activity was significantly less from LPS-stimulated whole blood treated with 1, 5, 10 and 25 ug/mL of PMB compared to blood not treated with PMB ($p\leq0.004$). However, although LPS-induced TNF production was blunted by PMB, it was still significantly greater ($p\leq0.01$) than TNF production from control at all concentrations tested with the exception of 25 ug/mL. At 25 ug/mL of PMB, LPS-induced TNF production was not significantly different from control ($p=0.17$).

In vivo Response to PMB Treatment

Physiologic parameters:

After LPS administration, rectal temperature increased significantly over the duration of the study ($p<0.001$) with a peak at 4 to 5 hours (Figure 2). None of the cats developed hypothermia. There was no significant temperature difference between treatment groups at any time point ($p=0.109$).

Respiratory rate was variable in all cats, ranging from 18 to 120 breaths per minute. There was no pattern of change over time or difference between treatment groups (data not shown). Of importance, despite the variability, none of the cats developed respiratory distress, open mouth breathing or cyanosis.

There was no significant difference in HR over time associated with LPS infusion or between treatment groups ($p=0.718$) at any time point. No cat developed bradycardia (HR < 140 beats/min). Lipopolysaccharide infusion did, however, induce a significant decrease in BP over the duration of the study compared to baseline ($p<0.001$), with no

significant difference between treatment groups (p=0.071) (Figure 3). None of the cats developed severe hypotension (BP < 60 mmHg).

The demeanor of all cats was considered bright, alert and responsive at the commencement of the study. LPS infusion resulted in a change in the demeanor of all cats, regardless of treatment group, varying from quiet, alert and responsive to lethargic. Neurologic evaluation remained normal in all cats throughout the study period regardless of treatment. All cats completely recovered normal demeanor by the following day. None of the cats developed clinical signs attributable to anaphylaxis. One cat in the placebo group developed diarrhea during the study period; experiencing 5 episodes between time 1.5 and 5.5 hours. None of the cats experienced vomiting.

CBC:

The white blood cell count (WBCC) significantly decreased (p<0.001) starting at one hour, reaching a nadir at 2 to 3 hours with recovery by 6 hours after LPS administration. The WBCC was significantly higher at 4 and 6 hours in the PMB treated cats compared to the placebo group (p=0.019) (Figure 4).

Plasma and urine biochemistry:

At 6 hours urine GGT was below the lower limit of detection (3 U/L) in all cats regardless of treatment group. There was no evidence of proteinuria, glucosuria, bilirubinuria or ketonuria in any cat regardless of treatment. Sediment examination was similarly unremarkable; all cats had an inactive sediment with no identifiable casts. The urine of all cats was isosthenuric (specific gravity 1.008 to 1.012).

TNF activity:

Plasma TNF activity was not detectable at baseline in any of the cats, regardless of treatment; therefore, this time point was removed from statistical analysis. Plasma TNF activity increased after initiation of endotoxin infusion in all cats with a peak at 1 hour in the placebo group and 3 hours in the PMB treatment group. The TNF activity was significantly lower in the PMB treated cats compared to the placebo group ($p<0.001$).

Discussion:

In this study, PMB was found to ameliorate proinflammatory sequelae of endotoxin challenge both *ex vivo* and *in vivo* in cats. *Ex vivo,* Cwb supernatant TNF activity was significantly less from LPS-stimulated whole blood treated with 1, 5, 10 and 25 ug/mL of PMB compared to blood not treated with PMB. *In vivo,* we evaluated the safety and anti-endotoxin effects of PMB in a clinically applicable, survival model of low-dose endotoxin infusion. As expected,[26] LPS administration induced significant systemic inflammation and hemodynamic derangement as indicated by the development of fever, leucopenia, increased TNF activity and relative hypotension. Polymyxin B administration in this model appeared safe and was found to blunt the LPS-induced leucopenia and plasma TNF activity. However, there was no significant difference between the placebo and PMB treatment groups in regards to rectal temperature, HR or BP.

The objective of the *ex vivo* study reported here was to investigate the pharmacodynamic properties of PMB using an LPS-stimulated whole blood culture system from adult cats to determine if and at which doses PMB could blunt TNF activity. Whole blood cultures have been validated in healthy humans as low-cost, surrogate measures of monocytic cytokine production.[153] TNF-α is a prototypic, early phase pro-

inflammatory mediator that has earned a position of prominence at the head of the inflammatory cytokine cascade because it can increase the expression of a wide variety of other pro-inflammatory mediators (e.g. nitric oxide and cyclooxygenase 2 of the arachidonic acid cascade), other cytokines (e.g. IL-1β, IL-6), chemokines (e.g. CXCL-8), and adhesion molecules, resulting in a cytokine storm.[154] In addition, TNF is involved in the hemodynamic derangement that accompanies sepsis. Infusion of TNF in experimental models results in hypotension, negative inotropy and clinical signs of shock. TNF also stimulates neutrophil release from bone marrow; the resultant early neutrophilia is one of the clinical criteria used in the diagnosis of sepsis. Another important consequence of TNF production includes resetting of the hypothalamic set point resulting in fever.[155, 156] Some studies have documented a correlation between plasma TNF concentrations and mortality in septic people.[157, 158]

Tumor necrosis factor production from LPS-stimulated feline Cwb has been previously reported;[148, 159, 160] however, to the knowledge of the authors, this is the first study to evaluate the effect of PMB on TNF production from endotoxin-stimulated feline Cwb. In our study, TNF production was significantly less from LPS-stimulated whole blood treated with PMB compared to blood not treated with PMB but remained greater than production from control except at 25 ug/mL PMB. These findings are consistent with what has been documented in an *ex vivo* model of endotoxemia in horses where PMB caused a significant dose-dependent decrease in endotoxin-induced TNF activity.[24] Similarly, treatment with low dose intramuscular PMB in a cecal ligation and puncture model of sepsis in rats attenuated TNF production from isolated Kupffer cells, *ex vivo.*[146] It should be noted that, while not significant (p=0.42), whole blood treated

with 25 ug/mL of PMB in the absence of LPS had more than twice the TNF production of whole blood treated with the other concentrations of PMB. Therefore, it is possible that the lack of difference between TNF production from LPS stimulated and control whole blood treated with PMB at 25 ug/mL was due to an increase in TNF production from the control blood as opposed to a lack of a true treatment effect from PMB. Polymyxin B stimulation of TNF production has been previously documented at higher concentrations of PMB (>20 ug/mL),[161] but not at 10 ug/mL.[118, 161] Documentation of a reduction in LPS-induced TNF activity by PMB subsequently prompted an *in vivo* study evaluating the anti-endotoxin effects of PMB. While there is no literature documenting a study which has correlated IV dose of PMB with concentrations in whole blood cultures, a dose of 1mg/kg of PMB was chosen to achieve a plasma concentration around 1 ug/mL in a 4.9kg cat with an estimated blood volume of 240 mL.[162] Higher doses were avoided to reduce the risk of toxicity and given the aforementioned stimulation of TNF production by high concentrations of PMB.[161]

In vivo, we chose to evaluate PMB in a low-dose endotoxin infusion model to avoid several disadvantages of high-dose endotoxin bolus models of sepsis, such as that used by Hughes et al., notably the induction of massive proinflammatory cytokine production leading to overwhelming inflammation, acute circulatory collapse and rapid mortality.[25, 163] Unlike the aforementioned model, the pre-clinical feline model using low-dose endotoxin not only has less severe clinical manifestations, but as a result also facilitates use of minimally instrumented conscious cats, thereby preventing anesthesia-induced interference with the neuro-endocrine axis and cardiovascular function. Compared with models of overwhelming sepsis resulting in rapid mortality, this model is

more likely to provide clinically useful data pertaining to novel sepsis treatments prior to evaluation in pet cats.

Altered body temperature is a common finding in cats with sepsis.[9, 138] Endotoxin infusion induces a febrile response in cats as was found in our study.[26] The etiology of fever in sepsis is likely associated with endogenous pyrogen production. In this study, PMB did not ameliorate the endotoxin induced fever, in contrast to PMB administration in studies of experimental sepsis in horses,[18] and naturally developing sepsis in humans.[147] The lack of a treatment effect in this study may be related to timing of administration in that body temperature had already increased by the time that PMB treatment was commenced (i.e. 30 minutes after the start of the endotoxin infusion). Barton et al. documented that pretreatment with PMB before the initiation of endotoxin infusion prevented LPS-induced fever, whereas horses receiving PMB after the onset of endotoxemia still developed fever, albeit less severe at peak, consistent with their overall conclusions that PMB was most beneficial to horses when given before the onset of endotoxemia.[18] Nonetheless, fever in isolation is not considered a deleterious manifestation of sepsis, and may actually be protective in the presence of bacterial infection.[164, 165]

Polymyxin B has been previously reported to have adverse effects on respiration in cats secondary to neuromuscular blockade, with respiratory depression occurring at doses of 3 mg/kg IV (18,000 U/kg), progressing to respiratory arrest at 5 mg/kg IV (30,000 U/kg).[124] Respiratory rate was highly variable in all cats of this study (treated and untreated), but no cat developed evidence of respiratory distress, change in respiratory pattern, or cyanosis. Respiratory rate is likely influenced by a variety of factors in the

setting of endotoxemia and sepsis in addition to the severity of pulmonary inflammation, including the presence of fever, discomfort and anxiety. Of important clinical relevance is that no adverse effects on respiration were observed in our study with the use of lower doses of PMB (1 mg/kg IV).

In this study PMB did not have an effect on the hemodynamic status of endotoxemic cats with no significant difference evident in HR or BP between treatment groups at any time point. While there was no change in HR over time, these cats failed to develop compensatory tachycardia in the face of systemic hypotension, which is suggestive of hemodynamic derangement. The inability of PMB to prevent acute phase systemic hypotension seen in our study is consistent with findings in dogs and cats treated with PMB (5 mg/kg IV over 30 minutes beginning one hour after IV bolus injection of LPS, and 5 mg/kg IV bolus one minute prior to endotoxin administration followed by a 30 minute infusion of an additional 5 mg/kg).[21, 25] Interestingly however, the second/late phase of LPS induced hypotension, which is a preterminal event in these lethal models of endotoxemia, was ameliorated in both the feline study,[25] and a subgroup of dogs treated with PMB-modified LPS.[21] Similarly, treatment of rabbits with PMB one hour after intraperitoneal *E.coli* injection resulted in higher blood pressure compared to placebo treated animals.[20] These studies taken together, suggest that the ability of PMB to ameliorate LPS-induced hemodynamic derangement, as well as other biologic effects, may be related to the timing of administration. Pretreatment of the LPS with PMB (i.e. mixing the two compounds together) prior to administration and pretreatment of the patient with PMB prior to LPS administration are likely to dramatically reduce the ability of LPS to activate the innate immune response by preventing lipid A interaction with

CD14 and TLR4, and thus preventing much of the inflammatory cascade and resultant hemodynamic instability that occurs in endotoxemia. While the aforementioned rabbit study did not pretreat with PMB,[20] it is likely that intraperitoneal bacterial injection rather than IV endotoxin dosing may result in a more gradual onset of endotoxemia, thus ensuring greater antiendotoxin efficacy of PMB in regards to prevention of downstream proinflammatory signaling. Unfortunately, prophylactic administration of PMB prior to the onset of endotoxemia and sepsis is not practical in naturally occurring sepsis in cats, reducing the clinical applicability of such data.

While an earlier report documented that high dose PMB has dramatic and beneficial effects on late phase hypotension, metabolic acidosis and survival in feline endotoxemia,[25] the dose used is high enough to induce neurotoxicosis, nephrotoxicosis, respiratory arrest, cardiovascular depression and histamine-mediated hypersensitivity.[17, 121-123] Concerns about the toxic effects of PMB have previously limited its clinical use, both as an antibiotic and for its antiendotoxin effects, in non-equine species. The toxicity of PMB results from its ability to bind to phospholipid membranes with the potential to disrupt cellular function in multiple organ systems.[121] Given the potential for severe side effects, the authors felt that it was vital to evaluate the safety of a low dose PMB treatment protocol before use in naturally occurring feline sepsis. Importantly, our study showed that low-dose PMB (1mg/kg IV) is safe with no differences in neurologic examination, RR and character, HR and BP between placebo and PMB treated endotoxic cats. Also, while urine GGT:creatinine ratios could not be performed because the urine GGT was below the limits of detection, the cats did not develop overt evidence of renal failure. Additionally, we did not observe signs of anaphylaxis or gastrointestinal

disturbance. Our findings are consistent with the dose dependent nature of PMB toxicity and results of previous studies in which low doses of PMB (1 to 2 mg/kg) maintain considerable antiendotoxin activity while avoiding adverse effects.[23, 119, 147] The dose of PMB (1 mg/kg IV over 30 minutes) was chosen for use in this study as it was below the toxic threshold for many species, and indeed the same appears to be true in cats.

The beneficial effects of PMB treatment in maintaining higher WBCC in our study are additional evidence of the antiendotoxin effects of PMB, but, interestingly, are in contrast to other studies in other species evaluating the antiendotoxin effects of PMB. For example, in experimental *Pasteurella multicoda* sepsis in rabbits, PMB treatment improved survival but did not modify the leucopenia.[145] Similarly, leukocyte numbers were unaltered by PMB treatment in both experimental canine endotoxemia,[21] and naturally occurring endotoxic shock in dogs.[119] Complete blood counts were not reported in the previous study evaluating the antiendotoxin effects of PMB in feline endotoxemia.[25]

Tumor necrosis factor is a prototypic proinflammatory cytokine and early phase mediator of systemic inflammation in sepsis. Only two other veterinary studies have used *in vivo* TNF concentrations to evaluate the anti-endotoxin effects of PMB.[18, 119] Similar to our study, these studies have documented reduced TNF production in septic patients treated with PMB. Administration of Polymyxin E (2 mg/kg IM q 12 hours for two doses) to dogs with naturally occurring, parvoviral-induced, endotoxic shock resulted in a significant reduction in plasma TNF concentrations compared to control dogs.[119] This finding is especially important since rising plasma TNF concentrations are associated with mortality in dogs with naturally occurring parvovirus-induced sepsis.[120] In an equine

model of endotoxemia, treatment with PMB, both before and after administration of endotoxin, significantly reduced serum TNF concentrations, compared to horses receiving saline placebo.[18]

Conclusion- These data indicate that administration of PMB after the initiation of endotoxin infusion is safe and results in an amelioration of the severity of endotoxin-induced leukopenia and TNF activity in this experimental model of feline sepsis. While PMB was safe and effective in blunting systemic inflammatory sequelae of LPS in this endotoxemia model, its clinical utility in cats with established sepsis is not known. A carefully designed, randomized, blinded, placebo controlled clinical trial, with close adverse event monitoring, evaluating the use of PMB in naturally occurring Gram negative feline sepsis would be the next step in evaluation of this therapy for clinical use.

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Chapter 3 – Conclusions and Future Directions

Sepsis is a serious problem in feline patients causing substantial morbidity and mortality.[9, 137] In cats, Gram negative bacterial infections are a common cause of sepsis.[9, 138-142] During Gram negative sepsis, endotoxin or lipopolysaccharide (LPS), the glycolipid component of the cell wall of Gram negative bacteria, is released into circulation resulting in a systemic inflammatory response that can lead to multiple organ dysfunction, and death.[12, 13] Despite the potentially devastating nature of sepsis in cats, little is known about sepsis in this species, and the existing literature consists predominantly of retrospective clinical investigations or lethal, experimental models of sepsis that do not replicate naturally developing disease.

Our group has recently developed a low-dose endotoxin infusion, survival, model of sepsis in cats in order to better understand the pathophysiology of sepsis in this species, and evaluate novel therapies prior to clinical trials in client owned animals.[166] This model is attractive in that it mimics naturally developing sepsis in minimally instrumented, conscious cats. Given that Gram negative endotoxin is one of the most potent stimulators of the sepsis cascade in cats, we have chosen to evaluate the potential therapeutic role of antiendotoxin strategies for the management of feline sepsis.

The introductory chapter of this thesis reviewed the preclinical and clinical literature in regards to antiendotoxin strategies, including polymyxin B (PMB), by sequentially evaluating potential points of intervention at each stage of LPS signaling through the innate immune response. Many of these strategies have shown promise in pre-clinical, small animal (rodent), models of sepsis, but in the opinion of the author,

have been inadequately evaluated in a clinical setting. While many of these antiendotoxin strategies hold promise for the treatment of sepsis in cats, PMB was chosen for initial evaluation since it has previously been shown to be efficacious in a lethal model of feline endotoxemia,[25] is already familiar to veterinarians given its use in horses with endotoxemia,[27] is readily available, and affordable.

In this study we hypothesized that PMB would ameliorate endotoxin-induced systemic inflammation and hemodynamic derangement with minimal adverse effects in our previously characterized, low-dose endotoxin infusion model of feline sepsis. Polymyxin B (1 mg/kg IV over 30 minutes) did indeed demonstrate antiendotoxin effects *in vivo*, notably decreasing peak plasma TNF activity ($p<0.001$) and increasing white blood cell count ($p = 0.019$), with no adverse effects. Given the apparent safety and antiendotoxin effects of PMB in this endotoxemia model, a carefully designed, randomized, blinded, placebo controlled trial evaluating the use of PMB in naturally developing Gram negative sepsis in cats is warranted. Prior to evaluation in a clinical setting, pharmacokinetic data will be evaluated.

Based on the literature review summarized in chapter 1, the evaluation of other antiendotoxin therapies seems warranted, in addition to further exploration of the potential therapeutic role of PMB. Of specific interest is to the author is the use of antibiotics that reduce endotoxin release, and other inhibitors of LPS, such as surfactin C and Eritoran. It is likely that a randomized controlled trial comparing the efficacy of different classes of antibiotics in cats with sepsis could be immediately implemented in a clinical trial setting given that it would be very much in keeping with standard of care. In contrast, pre-clinical evaluation of surfactin C and Eritoran in an endotoxemia model

would be ideal prior to use in client owned cats as the safety of these drugs in cats is not known. While the magic bullet therapy to cure sepsis will likely remain elusive, the use of antiendotoxin strategies in addition to standard of care (source control, antibiotics and aggressive supportive care) may result in improvement in morbidity and mortality from Gram negative sepsis in cats.

Figure i. TNF concentrations in feline whole blood culture after stimulation with control (□) or LPS (■) and treatment with polymyxin B, *in vitro*. Data are expressed as mean±SE. *Supernatant TNF activity was significantly less from LPS-stimulated whole blood treated with 1, 5, 10 and 25 ug/mL of PMB compared to blood not treated with PMB ($p \leq 0.004$).

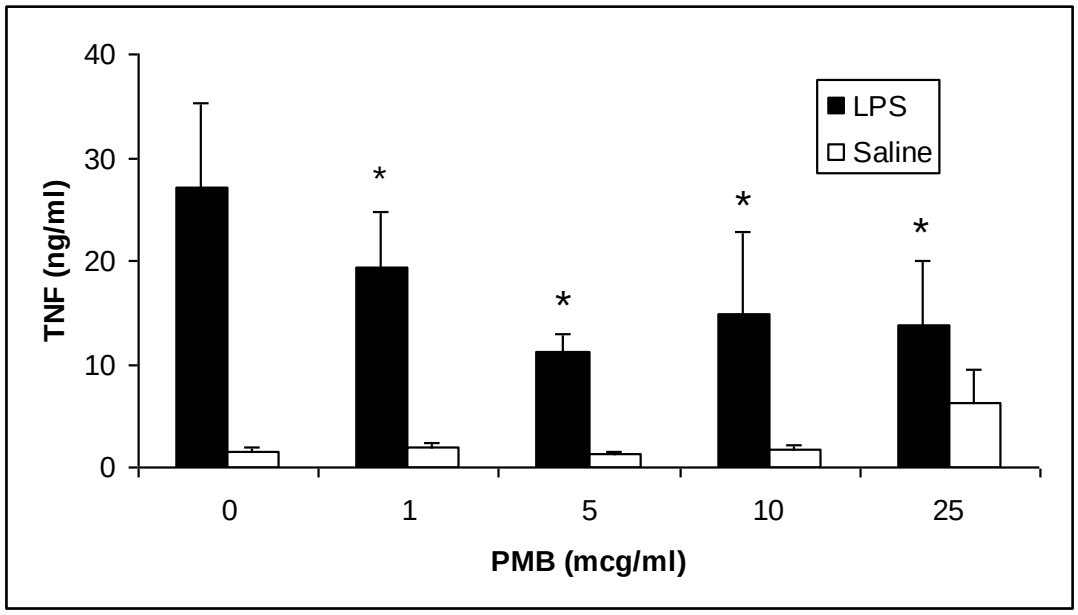


Figure ii. Comparison of rectal temperature between cats treated with polymyxin B (□) or placebo (■). Endotoxin infusion was initiated at time 0. Data are expressed as mean±SE. Temperature in both groups increase significantly over time compared to baseline. There was no significant difference between treatments.

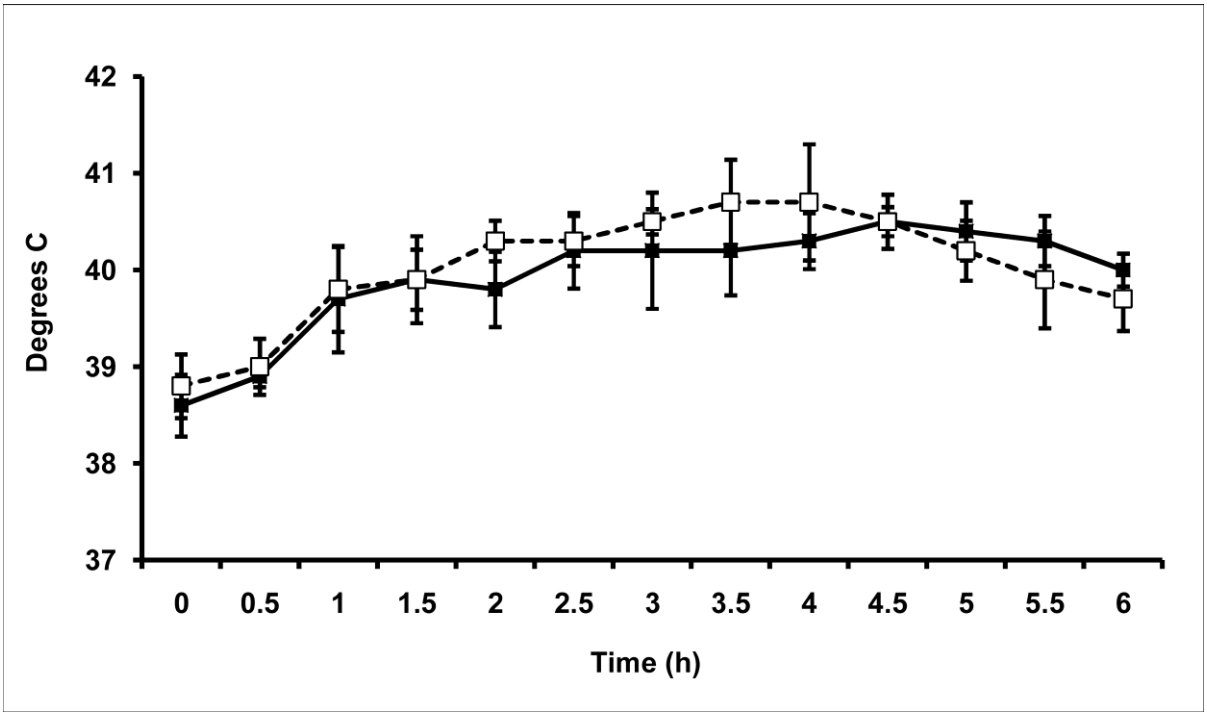


Figure iii. Comparison of systolic arterial blood pressure between cats treated with polymyxin B (□) or placebo (■). Endotoxin infusion was initiated at time 0. Blood pressure decreased significantly in both groups compared to baseline. There was no significant difference between treatments. Data are expressed as mean±SE.

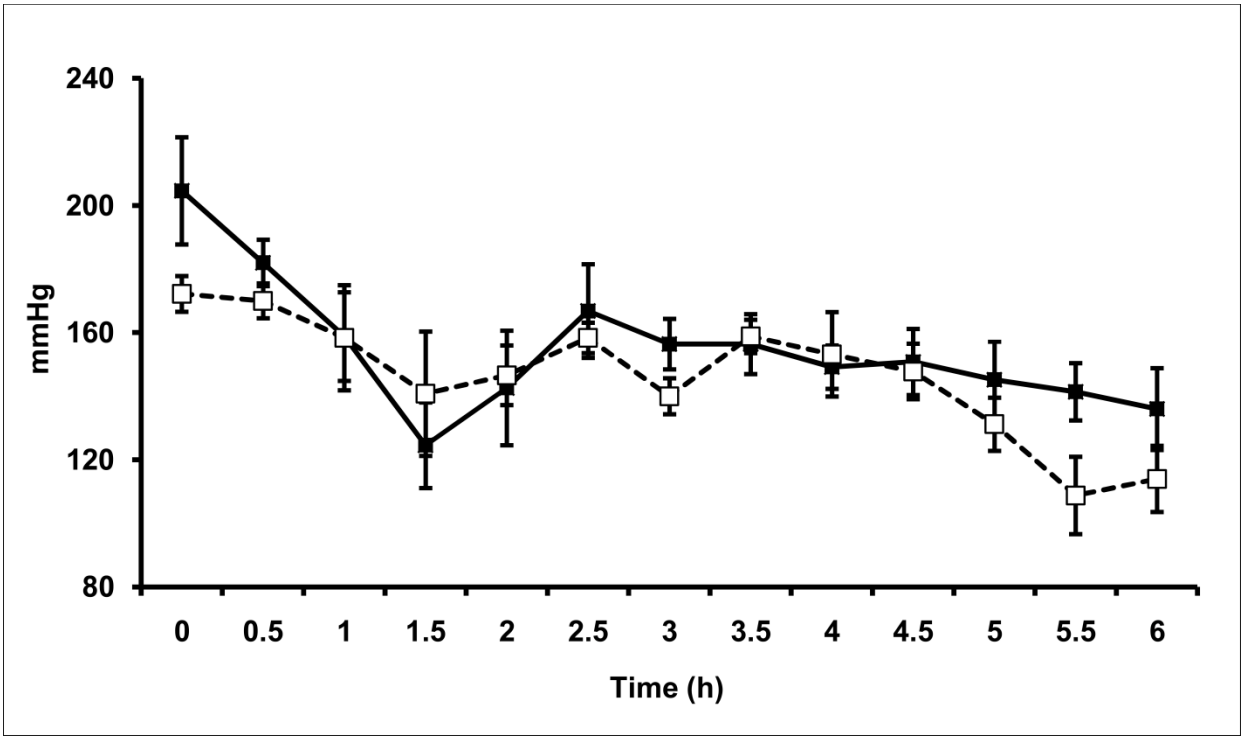


Figure iv. Comparison of white blood cell count between cats treated with polymyxin B (□) or placebo (■). Endotoxin infusion was initiated at time 0. Data are expressed as mean±SE. WBCC in both groups decreased significantly compared to baseline. * The WBCC was significantly higher at 4 and 6 hours in the PMB treated cats compared to the placebo group (p=0.019)

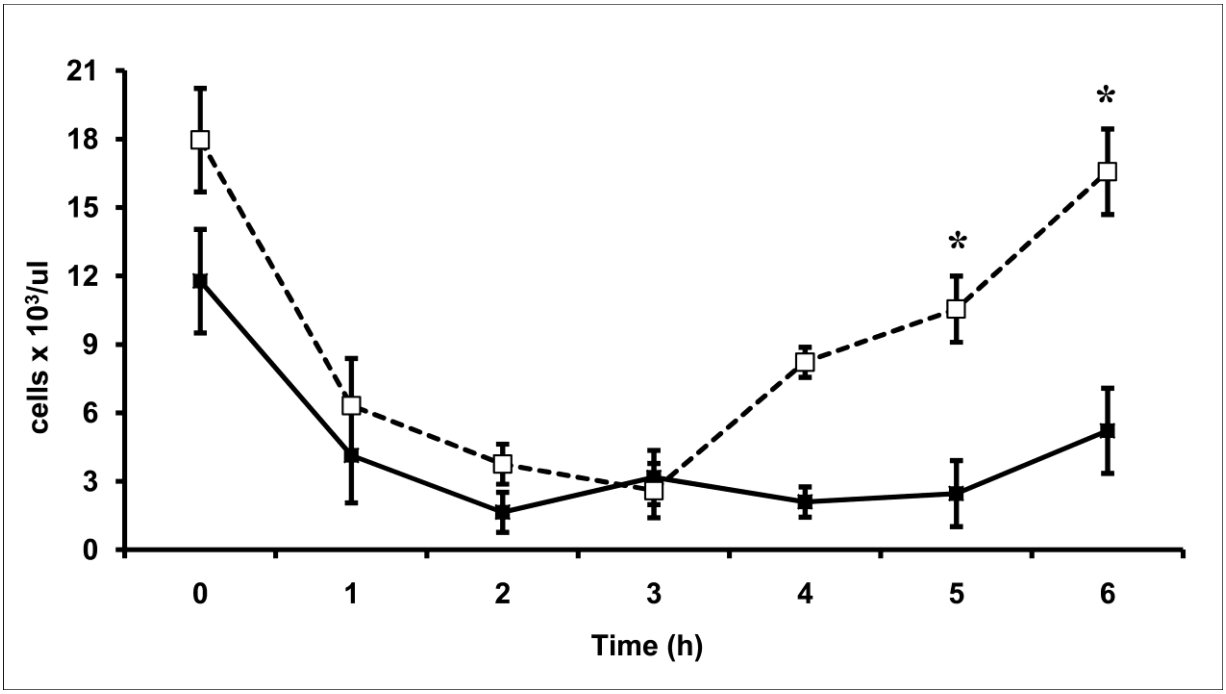
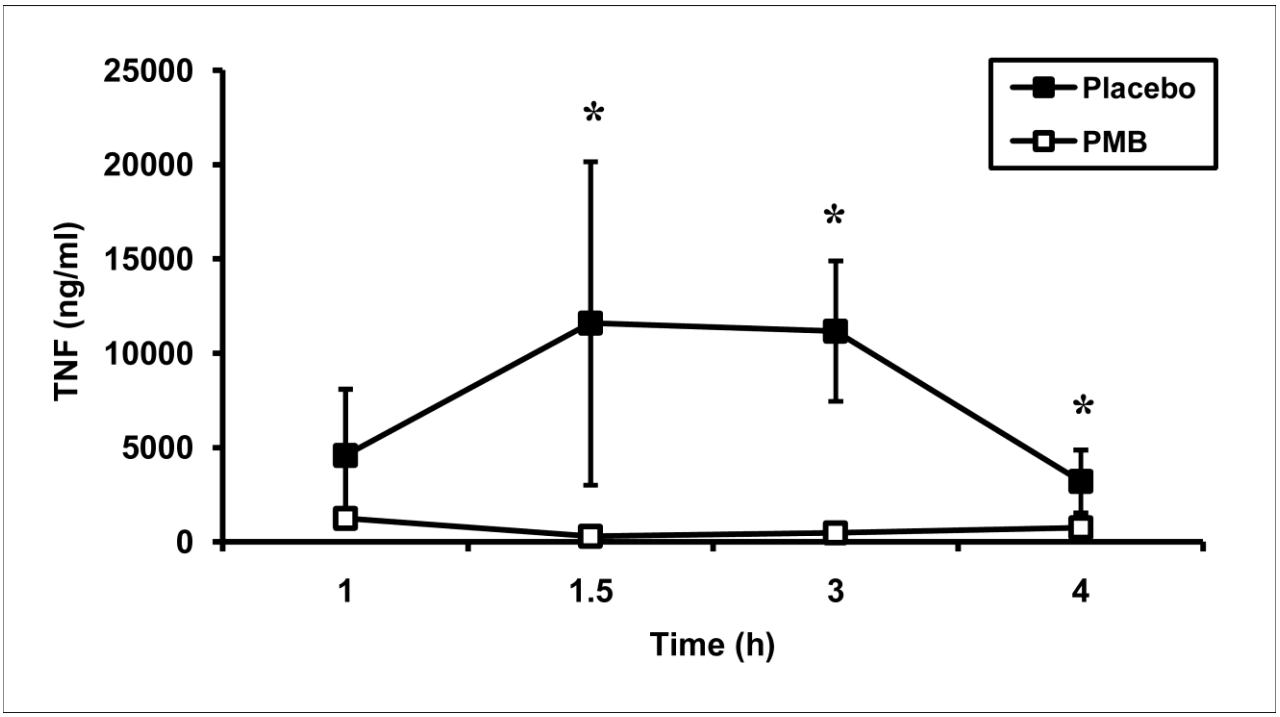


Figure v. Comparison of plasma TNF concentration between cats treated with Polymyxin B (□) or placebo (■). Endotoxin infusion was initiated at time 0. Data are expressed as mean±SE. * The TNF activity was significantly lower in the PMB treated cats compared to the placebo group ($p<0.001$).



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