EVALUATION OF PGG-GLUCAN, A NOVEL IMMUNOMODULATOR, IN *IN VITRO* AND *EX VIVO* MODELS OF EQUINE ENDOTOXEMIA

By

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Chairman: Martin O. Furr

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(ABSTRACT)

Justification - Endotoxemia is an important contributor to mortality and loss of use in the horse and results in significant losses to the equine industry on an annual basis.

Objective – To determine the effect of PGG-Glucan on the cytokine response to endotoxin in the horse.

Animals – Part 1; 6 adult horses. Part 2; 12 adult horses.

Procedure – Part 1; Whole blood was collected, aliquoted, and incubated *in vitro* in four groups; saline control, endotoxin (LPS) (100 ng/ml), PGG-Glucan (0.1, 1.0, 10 and 100 μg/ml) and LPS (100 ng/ml) plus PGG-Glucan (0.1, 1.0, 10 and 100 μg/ml). Supernatants were collected at 0, 6 and 12 hours and assayed for tumor necrosis factor α (TNFα) activity. Part 2; Horses received either PGG-Glucan (1 mg/kg) or an equal volume of isotonic saline (0.9% NaCl) IV over 15 minutes. Twenty four hours later blood was collected and mononuclear cells isolated for cell culture. Cells were treated

with LPS (100 ng/ml) and RNA extractions were performed at 0, 6, 12, 24 and 48 hours. Relative mRNA expression of TNFα, interleukin-1β (IL-1β), interleukin-10 (IL-10) and interferon-γ (IFN-γ) was determined by reverse transcription and real time polymerase chain reaction.

Results – Using an *in vitro* endotoxin challenge method PGG-Glucan altered the production of TNFα in a dose-dependent manner. PGG-Glucan had no effect upon the *ex vivo* cytokine mRNA expression of TNFα, IL-1β, IL-10 or IFN-γ.

Conclusions and Relevance – Although mild changes were observed in TNFα production *in vitro*, it is not likely that PGG-Glucan will have a significant effect upon clinical endotoxemia.

Dedication

Dedicated with thanks and appreciation to my parents who have always supported me in whatever endeavor I have chosen to pursue, and who have taught me that the world is bigger than what lies immediately before our eyes.

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List of Abbreviations

ANOVA	Analysis of variance
BPI	Bactericidal/permeability increasing
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
G3PDH	Glyceraldehyde-3-phosphate dehydrogenase
G-CSF	Granulocyte-colony stimulating factor
HDL	High density lipoprotein
IFN-γ	Interferon gamma
IL	Interleukin
IM	Intramuscular
IRAK	Interleukin-1 receptor-associated kinase
IU	International units
IV	Intravenous
KCl	Potassium chloride
LBP	Lipopolysaccharide binding protein
LPS	Lipopolysaccharide
LT	Leukotriene
mCD14	Membrane bound CD14
mRNA	Messenger ribonucleic acid

MTT	3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide
NaCl	Sodium chloride
$NaHCO_3$	Sodium bicarbonate
NF-κB	Nuclear factor-κB
NSAID	Non-steroidal anti-inflammatory drug
PAF	Platelet activating factor
PCV	Packed cell volume
PGG-Glucan	β-(1,6)-branched β-(1-3)-linked glucan
ROS	Radical oxygen species
RNA	Ribonucleic acid
sCD14	Soluble CD14
SIRS	Systemic inflammatory response syndrome
SR-A	Type A macrophage scavenger receptors
SQ	Subcutaneous
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TRAF	Tumor necrosis factor-associated factor
TSP	Total solids protein

Equine Endotoxemia – Literature Review

Endotoxemia is a major cause of loss to the equine industry on an annual basis and is a significant cause of mortality and morbidity in both neonates and adult. It has been recognized as a clinical syndrome in the horse for nearly forty years[1] and the financial burden of the disease to the equine industry is high, although the actual cost is difficult to accurately calculate.

Definition.

Endotoxemia refers to the presence of endotoxin in the blood. The term is also used to refer to the clinical syndrome that occurs as a result of overwhelming of clearance mechanisms and over activation of the body's normal defense mechanisms.[2-4] The term endotoxin refers to the lipopolysaccharide (LPS) portion of the outer cell wall of gram-negative bacteria. Lipopolysaccharide is a more accurate description of the molecule than the term endotoxin. Whereas endotoxin represents a more historical description referring to early work which suggested that the toxin was within the bacterial cell, as opposed to an exotoxin. This is not strictly the case. For the purposes of this review hereafter the term LPS will be employed in reference to endotoxin, while endotoxemia will be used to refer to the clinical syndrome.

Lipopolysaccharide consists of three structural components, an inner hydrophilic lipid A portion, a core oligosaccharide and an outer O-specific polysaccharide, shown

below in figure 1.[5] The lipid A component is very similar among bacterial species and, due to its hydrophobic nature, is largely buried within the cell membrane. It is generally accepted that lipid A is responsible for most of the toxic effects seen following LPS exposure[5-7] although the O-specific polysaccharide and core protein may also be important.[8,9] The O-specific polysaccharide consists of repeating chains of oligosaccharides that vary between bacterial species conferring the serological identity of an individual species. The O-specific portion is connected to the core oligosaccharide which, similarly to lipid A, is well conserved between species.[5] Enzymatic errors in the production of the O-specific portion result in the generation of "rough" mutant bacterial strains that lack the O-specific portion. This exposes the core protein to the immune system and is important in the generation of cross reactive anti-LPS antibodies.[6,10]

Lipopolysaccharide is released in large amounts during periods of bacterial multiplication or death and may be released as a free form or in combination with other bacterial surface proteins.[4,11,12] Free, soluble LPS is over 20 fold more biologically active than LPS bound to the cell wall[13] and while there are also many other bacterial products present in gram-negative bacteria capable of eliciting a profound immune response, there is convincing evidence that LPS is the most potent of all the cellular products.[4,14]

Importance

In adults endotoxemia is commonly seen as a sequel to many primary diseases, including colic, colitis, metritis and pleuropneumonia.[5] Colic remains the leading cause of death in the horse, accounting for over one quarter of all deaths,[15] with mortality rates for surgical colic approaching 50%, or greater, commonly reported in the literature.[16-22] However, mortality amongst this group of animals has been correlated with clinical and clinicopathological signs of endotoxemia.[23-26] In addition the prognosis for many cases is determined by the potential for the development of life threatening sequela[27-29] such as laminitis[30,31] and disseminated intravascular coagulation.[25,32-34]

Increases in admission and intra-operative endotoxin levels have been associated with increased mortality in horses.[33,35,36] In humans persistently high levels of circulating endotoxin have been correlated with the development of multiple organ failure and death.[37-39] Clinical and clinicopathological signs of endotoxemia such as tachycardia, hyperlactemia, abnormal mucous membrane color and refill time, and hemoconcentration have been evaluated as prognostic indicators prior to colic surgery and have been shown to correlate with increased mortality.[17,22,24,40-49] Additionally, several studies of horses clinically affected by gastrointestinal disease have reported that 25 to 44% of affected animals had circulating endotoxin detected in the blood[25,26,35,36,50,51] or peritoneal fluid.[51,52] Given the short half life of endotoxin (less than 2 minutes during the first phase of elimination following intravenous injection in the horse)[50,53] it is likely that the proportion of affected animals detected in these studies significantly underestimates the

prevalence of endotoxemia in this population. The persistence of cardiovascular signs following clearance of circulating endotoxin is further supported by experimental challenge in human volunteers where a small dose of endotoxin results in cardiovascular changes that persist for hours, although the endotoxin is rapidly undetectable.[39]

Increased circulating levels of endotoxin have been demonstrated following experimental small intestinal strangulation[54] and circulating endotoxin has been detected in 85% of horses with carbohydrate-overload induced laminitis.[55] Other studies have reported an increase in caecal endotoxin concentrations associated with the development of laminitis[31] further strengthening the association between the two disease conditions.

Neonatal mortality also contributes significantly to losses in the equine industry and historically mortality rates as high as 3.6% in the first week of life have been reported.[56] Septicemia is the most common acquired disease in foals and has the lowest survival rate of the acquired neonatal diseases.[57,58] Mortality of foals with septicemia is high, ranging from 25 to 74% in clinical studies, depending on the entry criteria,[57-60] and approximately one third of deaths in thoroughbred foals less than 3 months of age are attributable to septicemia.[61] The connection between septicemia and endotoxemia is strong and it is well recognized that, although other antigens including gram-positive bacteria, can produce similar systemic responses,[62,63] endotoxemia associated with gram-negative septicemia contributes significantly to the high mortality rates of neonatal septicemia.[64] In one review of 47 foals affected by septicemia, gram-negative organisms were isolated from all foals[58] and in another study 93% of affected foals had gram-negative organisms isolated.[59] The clinical and clinicopathological signs of septicemia

and endotoxemia are similar and experimental studies involving administration of endotoxin have reproduced the clinical and laboratory findings of septicemia in foals.[59,65-69]

Sources of Lipopolysaccharide (LPS).

In the normal horse large numbers of gram-negative bacteria, and therefore large amounts of LPS, reside in the large intestine.[26,31] Tight mucosal junctions between epithelial cells, secretions from epithelial cells and resident bacteria all contribute to an effective defense mechanism that prevents absorption of significant amounts of LPS into the circulation.[5,7,9,26] Increased absorption may occur in animals with concurrent systemic, gastrointestinal or liver disease.[6,70-75]

When small amounts of LPS are absorbed into the portal vein some is neutralized by circulating anti-LPS antibodies.[76] The remainder is bound on specific proteins (LPS-binding protein (LBP)).[77] Under physiological conditions LPS bound to LBP is then cleared by the liver, predominately by the mononuclear phagocytic system.[71,78,79] In rats, Kupffer cells are responsible for approximately 80% of the liver's clearance capacity for LPS with endothelial cells clearing the remainder, although the relative contribution of each is dependent on the dose of LPS. Clearance occurs by fluid phase endocytosis and appears to be saturable with higher doses of LPS having a significantly longer half life.[79] The presence of low, systemic, circulating levels of LPS in the horse was initially thought

to be negligible,[6] however with improved assay techniques low levels of circulating LPS has been demonstrated in normal animals.[35]

Increased amounts of LPS may enter the systemic circulation by one of several ways. Firstly, if the amount of LPS entering the portal circulation is large enough, the clearance capacity of the liver is overwhelmed leading to some LPS effectively escaping the liver into the systemic circulation. This commonly occurs secondary to a wide variety of intestinal conditions that compromise blood flow to bowel, including many surgical gastrointestinal conditions, severe colitis and hypovolemia. Alternatively, LPS that originates from sites in the body other than the gastrointestinal tract, such as the peritoneal cavity, pleural cavity or uterus, may bypass the portal circulation and directly enter the systemic circulation.[54,80] Although the thoracic duct has not been confirmed to be an important portal bypassing conduit for peritoneal LPS in horses, it is well recognized as such in other species[72,76,81-83] and may, in fact, be responsible for most of the increase in circulating levels of LPS under certain disease conditions.[72] Animals with severe intestinal disease, such as strangulating obstructions, may have entry of LPS into the systemic circulation due to a combination of both mechanisms.[82] Experimental models have shown increased levels of LPS in the peritoneal fluid of ponies with strangulating small intestinal lesions and ischemic damage to the mucosa allows increased absorption of LPS into the portal circulation.[80]

When housed in traditional indoor management systems horses may also be exposed to sufficiently high enough levels of LPS via inhalation to contribute significantly to pulmonary disease, without systemic effects.[84] Other situations in which

significant increases in circulating levels of LPS have been reported include immediately after racing in horses,[85] during severe heat stress in primates (body temperature greater than 41.5^{o}C)[76,86-89] and in humans with liver disease.[71,90,91]

Cellular Activation.

The Innate Immune Response.

The myriad of clinical and laboratory abnormalities seen in endotoxemia result from activation of the innate immune response and the production of a wide range of inflammatory mediators, of which over 150 have been described.[3] The innate immune system is vital to the immune response and represents the front line defense to pathogens. It is largely mediated by white blood cells that phagocytose and kill bacteria, and initiate the pro-inflammatory immune response. The innate immune system must differentiate pathogens from self and is also important in the adaptive immune response and presentation of components of pathogens to T-cells.[92]

It is important to recognize that the mechanisms and mediators that contribute to the development of shock, multiple organ disease, subsequent organ failure and death are fundamental, important components of the innate immune system. The clinical manifestation of endotoxemia represents over-activation of the body's normal, vital protective mechanisms.[4] In fact mice that are resistant to LPS due to various genetic mutations are dramatically more sensitive to lethal infection from gram-negative organisms. Most notably the 50 % lethal dose of *Salmonella typhimurium* was less than two organisms in LPS non-responsive mice compared with over 1,000 organisms in normal animals underlying the importance of an appropriate immune response.[93]

The series of events that leads to the activation of the innate immune response following exposure to LPS is not completely elucidated, however numerous important factors, such as lipopolysaccharide binding protein, CD-14, toll-like receptors and ancillary proteins, such as MD-2, have been described and a review of the current proposed pathway is provided below.

Lipopolysaccharide Binding Protein (LBP).

Lipopolysaccharide binding protein is a 60-kDa serum glycoprotein first described in 1986[94] that is present in the serum of normal animals and humans.[95] It is important in facilitating the binding of LPS to the CD14 receptor which is responsible for the initiation of cellular mechanisms (via a toll like receptor (TLR4)) and up regulation of the production of a wide variety of cellular products. Lipopolysaccharide binding protein is capable of binding LPS as well as LPS-containing particles, including intact bacteria, and serves both as a transport protein for LPS and as a vital component in the cellular recognition of LPS.[96,97] Lipopolysaccharide binding protein is well conserved between mammalian species and binds LPS via the lipid-A component.[98] Although several plasma proteins interact with LPS, the binding of LPS with LBP and the subsequent interaction with the CD14 receptor is unique to LBP.[99]

Absence of LBP lowers the sensitivity of monocytes to LPS by approximately 250 fold compared with concentrations normally found in plasma.[96] Lipopolysaccharide binding protein deficient mice are resistant to the toxic effects of LPS following

intraperitoneal administration.[100] Over 100 fold lower production of tumor necrosis factor α (TNFα) in LBP-deficient mice, and absence of mortality, compared with mortality of over 90% in the control group, has been demonstrated.[100] However, the same series of studies reported greatly increased mortality from gram-negative bacterial challenge in LBP-deficient mice. The absence of LBP resulted in decreased bacterial clearance and killing, and 10 fold higher numbers of organisms in the peritoneal cavity, liver and spleen at 48 hours following live bacterial challenge.[100] This increased susceptibility does not appear to be related to decreased clearance of LPS as LBP-deficient mice clear LPS at a similar rate to that of normal mice.[100] The binding of LPS and the subsequent cytokine release by neutrophils and macrophages *in vitro* has been shown to be enhanced by addition of low concentrations of LBP.[101-103] The binding of LBP to CD14 does not occur in the absence of LPS conferring a dual dependency within the system.[97,104] Lipopolysaccharide binding protein binding is specific for mononuclear phagocytes as LPS-LBP complexes does not bind with other cell types.[97]

The series of studies described above provides convincing evidence that the presence of LBP is an important component of the innate immune response to LPS. On the other hand, Wurfel *et al* (1997)[105] have recently challenged the previously held view that LBP is an essential component of the immune response to LPS. The basis for this is that, although depletion of LBP in *in vitro* whole blood models decreased TNFα production by over 1,000 fold, the findings were not reproducible *in vivo*.[105] Thus an alternative pathway for macrophage stimulation may exist in the intact animals, however

most authors still consider LBP an important and non-redundant component of the innate immune response under physiological conditions.

Whereas depletion of LBP is associated with decreased responsiveness to LPS stimulation, so too is exposure to high serum concentrations. Lipopolysaccharide binding protein is an acute phase protein synthesized by the liver and dramatic elevations in serum concentrations, 30 to 100 fold, can occur during the early response to insult.[77,96,106,107] Stimulus for increased production includes trauma, the systemic inflammatory response syndrome (SIRS) and sepsis. Increased LBP production can result from stimulation of hepatocytes by IL-1 alone, or synergistically by IL-1 and IL-6.[107] The maximum response is observed at 1 – 3 days and LBP levels appear to be a relatively sensitive indicator of sepsis when compared to other acute phase markers.[96,107] Persistently high concentrations of LBP have been associated with a poor prognosis, although this observation may merely reflect persistent up regulation of the innate immune system rather than a causative effect.[101,108]

Acute phase concentrations of LBP inhibit the effects of LPS *in vitro*. Likewise, intraperitoneal injection of high concentrations of LBP protected mice from otherwise lethal levels of LPS and live *Escherichia coli in vivo*. Interestingly this protective effect was not observed following intravenous injection of LBP.[101] It has been proposed that high concentrations of LBP may enhance CD14 mediated phagocytic uptake of gram-negative bacteria *in vivo*[102] despite evidence of reduced uptake of bacteria *in vitro* at high concentrations of LBP.[101] One reason for the discrepancy of these findings may be that

the ability of LBP to facilitate removal of LPS is more dependant on the LPS/LBP ratio than absolute concentrations.[101,109]

In addition to interacting with the CD14 receptor, LBP facilitates the removal of LPS from the circulation by enhancing neutralization by high-density lipoproteins (HDLs). This primarily occurs via a two step process which is catalyzed by LBP. There is an initial transfer of LPS from the LPS-LBP complex to sCD14 followed by the transfer of LPS from the LPS-sCD14 complex to HDL.[110] Importantly, kinetic studies have shown that binding with CD14 receptors occurs prior to neutralization by HDLs.[107] Lipopolysaccharide binding protein also enhances the direct binding of LPS to HDLs.[111]

The binding of LPS to HDLs results in increased LPS clearance, decreased LPS binding to cells and subsequently decreased cellular production of pro-inflammatory cytokines.[112,113] The ability of HDLs to down regulate the innate immune response has been demonstrated in a murine study where doubling of plasma HDL concentration by genetic manipulation resulted in a 3 – 4 fold increase in survival following LPS challenge and, similarly decreased survival in transgenic mice with low HDL concentrations.[114] In the same series of studies the administration of reconstituted HDLs also resulted in improved survival.[114] Low density lipoproteins are also capable of binding LPS, possibly with a higher affinity than HDLs.[113] The administration of triglyceride rich lipoproteins has been shown to decrease mortality due to septic shock in rats.[112]

Overall LBP plays a central role in both the initiation of the innate immune response as well as clearance of LPS and down regulation of the acute phase response.

This effect is likely aimed at an early, effective immune response while preventing over stimulation and subsequent over activation of the inflammatory cascade leading to shock.

In addition to LBP several other families of acute phase proteins, including bactericidal/permeability increasing proteins (BPI), have been described.[107,115,116] Bactericidal/permeability increasing proteins are immunologically and structurally similar to LBP but have clearly different origins, being produced by neutrophils during differentiation, and functions.[77,117] These proteins have a much higher affinity for LPS than LBP and by binding LPS make it less biologically available to cells, thereby blocking its effect.[107,118] Bactericidal/permeability increasing proteins also facilitate the aggregation and sedimentation of LPS complexes, further contributing to its clearance.[109] In comparison LBP causes disaggregation of LPS clusters and increased biological activity.[109] Isolated BPI has been shown to rapidly bind to the lipid-A component of LPS within gram-negative organisms and kill these organisms. In comparison, LBP has no ability to kill bacteria, even at high concentrations, and it appears that despite the immunological similarity that functionally the only property shared by LPB and BPI proteins is the ability to bind LPS.[77]

CD14 Receptors.

CD14 receptors also play a vital role in initiation of the cellular response and although they lack transmembrane and intracellular domains, and thus are not directly responsible for cellular activation, they facilitate the activation of toll-like receptors by

LPS.[119] CD14 negative mice are dramatically less responsive to LPS stimulation than normal mice,[120] whilst transgenic mice expressing CD14 are sensitized.[121] CD14 likely facilitates the transfer of LPS from the extracellular space to the membrane where it can interact with a number of receptors.[119] CD14 is also capable of binding and signaling internalization of whole gram-negative bacteria by a LBP dependent pathway, suggesting that free endotoxin is not required for CD14 recognition.[102]

CD14 exists in two forms, a membrane bound form, mCD14, and a soluble form, sCD14.[4] Membrane bound CD14 is present at the surface of myelomonocyte cells and has two functions as a result of acting as a cellular receptor for LPS at the membrane surface. Receptor signal transduction and activation of the host cell, and internalization of the LPS are both mCD14 mediated.[4] The duplicity of mCD14s function is demonstrated by the use of various forms of monoclonal antibodies against mCD14 where some block cellular activation while allowing internalization while others have the opposite effect.[104] The ability of CD14-positive cells to internalize LPS appears to be due to independent scavenger receptors, discussed later.[104] The binding of the LPS-LBP complex varies depending on the receptor type: where binding with sCD14 results in transfer of LPS to the sCD14, binding with mCD14 is more physically stable though the exact mechanism is unclear.[104,110]

Membrane bound CD14 mediated LPS internalization occurs within minutes of exposure to low concentrations of LPS and is dependent on the number of LPS aggregates. The rate of internalization increases as the number of aggregates increases.[122,123] Conversely, the rate of cellular activation does not increase as the

number of LPS aggregates increases. This suggests that the process may represent another mechanism of enhanced LPS clearance at high concentrations that prevents over activation of the immune system.[123] The exact role of internalized LPS in cellular activation is unclear.[4] The importance of mCD14 in cellular activation has been verified by a number of studies.[120,121,124,125] The use of anti-CD14 antibodies and mice deficient in CD14 receptors has demonstrated decreased physiological responsiveness to LPS.[120,124] The transfection of CD14 negative cells with CD14 greatly enhances the cellular response.[121,125]

Soluble CD14 results from either the shedding of mCD14 by monocytes,[126] or the production of CD14 molecules lacking the glycerophosphate inositol that secures mCD14 within the cell membrane.[4] Soluble CD14 can bind to LPS-LBP complexes and the resultant complexes may then activate CD14 negative cells, such as endothelial and some epithelial cells.[127,128] The activation of non-CD14 expressing cells by LPS-LBP-sCD14 complexes may result in cellular cytokine production[128] or it may be a cellular mechanism for clearance of LPS via internalization.[127] The exact role of sCD14 in activation of endothelial cells remains unclear as activation of CD14 negative cells in whole blood studies preferentially occurs by indirect pathways utilizing mCD14 on monocytes and secretion of a soluble mediator or mediators, such as TNF and interleukin-1 (IL-1), without sCD14 involvement.[129] Furthermore, it is unclear whether the LPS-LBP-sCD14 complex binds to the cell membrane or whether activation occurs by another mechanism.[127] The ability of sCD14 to opsonize bacteria may help mobilize LPS onto mCD14 and thus upregulate the cellular response of CD14 positive cells, in a

LBP independent manner.[130,131] Furthermore sCD14 may help permeabilize bacterial membranes to antimicrobials.[132]

Soluble CD14 may also play an important role in down regulation of the immune response to LPS by a number of mechanisms. In a manner similar to acute phase concentrations of LBP, high concentrations of sCD14 lower mortality in mice exposed to LPS.[133] At high concentrations sCD14 inhibits LPS-LBP induced activation of CD14 positive cells, presumably by a competitive action with mCD14, although this effect occurs only at supraphysioloical levels of sCD14.[134] As discussed above, sCD14 also facilitates the transfer of LPS to HDLs allowing removal of LPS from the circulation.[4]

Other Membrane Bound Receptors.

Other membrane bound receptors, including the CD11/18 receptor molecule and type A macrophage scavenger receptors (SR-A), may also be important in the cellular response to LPS. CD11c/18 is capable of initiating bacterial internalization, intracellular signaling and phagocyte activation[135] and, although it is not vital in the initiation of TNF production, it likely plays an important role in optimizing production of cyclooxygenase-2 and other mediators.[136]

Type A macrophage scavenger receptors molecules are expressed by many macrophage populations, including gut and Kupffer cells, and have been demonstrated to be important in the macrophages ability to recognize, bind, internalize and partially break down LPS.[137] Type A macrophage scavenger receptor knock out mice are more sensitive

to LPS challenge with increased production of pro-inflammatory cytokines and increased mortality observed following challenge.[138] The protection provided by SR-A may be mediated through binding and internalization of LPS, thereby decreasing the amount of LPS available to bind with CD14. Type A macrophage scavenger receptor knock out mice, pretreated with liposomes to eliminate Kupffer cells and other macrophages, have decreased ability to clear LPS.[138] Furthermore, LPS stimulated macrophages express high levels of SR-A consistent with a inducible, negative feedback mechanism.[138]

Toll-Like Receptors (TLR).

CD14 lacks an intracellular domain and transmembrane proteins and thus is incapable of directly activating cellular cytokine production. Instead, CD14 facilitate the transfer of LPS to toll-like receptors (TLRs) which are then responsible for intracellular signaling.[139] Of the TLRs, TLR4 appears to be the most important in the innate immune response to LPS.[4,119] Evidence for the vitality of the TLR4 in the response to LPS is confirmed by observations that TLR4 knockout mice are resistant to the effects of LPS,[140] while other mice, specifically C3H/HeJ and C57BL/10ScCr types, which are non-responsive to LPS have mutations in, or deletion of, the TLR4 domain.[141] Similarly hyporesponsiveness of humans to LPS has been associated with point mutations in the TLR4 domain.[142] Furthermore, transfection of TLR4 into specific strains of human embryonic kidney cells that are unresponsive to LPS restores an appropriate response.[143]

It is unclear at this stage whether other TLRs are involved in LPS signaling under physiological conditions.[4,144] It is likely that TLR2 is responsible for the recognition of gram-positive bacteria but that some of the early work that suggested that TLR2 was important in LPS recognition was inaccurate as efforts to repeat the findings have not been successful. Alternatively, TLR2 appears to act as a LPS receptor when overwhelmed by excessive concentrations *in vitro*.[106,144] Further evidence for the lack of a vital role for TLR2 in LPS recognition is that macrophages lacking TLR2 receptors *in vitro*, and Chinese hamsters lacking TLR2 receptors *in vivo*, are capable of launching a normal immune response to LPS.[145,146]

MD-2.

In addition to CD14 and TLR4 another protein, termed MD-2, is required for LPS activation of cells.[147,148] MD-2 is a secreted 20 -30 kDa glycoprotein lacking a transmembrane domain that is physically associated with TLR4 and CD14 to form a ternary complex which, in the presence of LPS, is capable of signaling cellular activation.[139,149] The presence of a point mutation in the MD-2 domain rendered hamster cell lines transfected with human CD14 unresponsive to LPS, while transfection of this mutant cell line with wild type MD-2 restored an appropriate response.[150] MD-2 may exert this effect by changing the structure of TLR4 affecting its affinity for LPS, or it may function as part of a true TLR4 ligand.[150] MD-2 is capable of binding LPS directly. Its effects on the immune system are concentration dependant with low concentrations

being inmmunostimulatory and higher concentrations being immunosuppressive via direct, competitive binding of LPS. In a manner similar to that of sCD14, this later effect may only be relevant at supraphysiological levels.[151] MD-2 may also be responsible for the ability of LPS to stimulate an immune response in the absence of LBP.[151,152] MD-2 can associate with other members of the Toll-like receptor family and by doing so confers increased responsiveness to pathogens other than LPS.[147-149] It is possible that another, as yet unidentified, receptor may be required for completion of the LPS signal transduction pathway.[150]

Although CD14, TLR4 and MD-2 are clearly important in the immune response to LPS some authors have recently questioned whether they are an absolute requirement, or whether clusters of other cellular receptors can perform the same function.[119,153] Some evidence exists to suggest that the response to LPS at low concentrations is CD14 and TLR4 dependent while higher concentrations of LPS can induce cellular activation via alternative pathways.[120,136] The validity of this theory remains to be determined at this stage. Given the rapidly changing nature of the field it is likely that many alternative pathways, or combinations of current pathways, will be discovered.

Toll-Like Receptor Signaling Pathways.

Lipopolysaccharide intracellular signaling through the LBP-CD14-TLR4-MD-2 system is a very complex process and detailed discussion is beyond the scope of this review. Interestingly, TLR signaling appears, at least in part, to occur by an internal

manner very similar to IL-1 signaling. The initial response involves the recruitment of the adaptor molecule MyD88 and IL-1 receptor-associated kinase (IRAK).[154] MyD88 is also a Toll-like molecule and binds to TLR4 complex by a Toll-Toll interaction.[4,155] Absence of MyD88, due to genetic deletion, results in impaired responsiveness to LPS in mice confirming its importance in cellular signaling.[156] The binding of MyD88 is followed by recruitment of IRAK to the complex. IRAK then dissociates from the complex and activates two separate inflammatory cascades via tumor necrosis factor receptor-associated factor 6 (TRAF6).[4] The importance of TRAF6 has been demonstrated in similar manner to MyD88 by hyporesponsiveness to LPS in TRAF6 deficient mice.[157]

Of the signal transduction pathways the nuclear factor-κB (NF-κB) pathway is the best described and understood. Activation of the NF-κB pathway results in activation of IκB kinases, phosphorylation of IκB followed by proteolytic degradation and subsequent dissociation of IκB from NF-κB, and ultimately translocation of NF-κB into the nucleus.[132] Translocation of NF-κB into the nucleus results in the induction of gene transcription leading to cytokine production.[132] The importance and details of the other pathways are only just beginning to be fully elucidated and discussion of them is beyond the scope of this review.

Products of Cellular Activation.

Cytokines, lipid derived mediators and coagulation or fibrinolytic factors are the three main classes of substances produced as a result of the cellular activation of monocytes and macrophages. These products, either directly or indirectly, are responsible for the vast array of systemic responses that are observed in endotoxemia.[5] These products interact with multiple cell types and are ultimately responsible for the generation of numerous other cytokines and products that further stimulate and regulate the inflammatory response.

Cytokines.

Cytokines are a group of soluble proteins released by a variety of cells, including mononuclear phagocytes, that affect the regulation and activities of, and interactions between, other cells and tissues. They are capable of both systemic and local effects and are produced by a variety of cells, under both physiological and pathological conditions.[3] Tumor necrosis factor alpha and IL-1 are key mediators in the inflammatory cascade following exposure to LPS and have been the most extensively studied.[3] Tumor necrosis factor α has been described as the principle mediator in endotoxic shock.[158] In humans with sepsis TNFα levels have been correlated with both severity of disease[159,160] and with outcome.[161,162] The actions of IL-1 are similar to, though generally less pronounced, than TNFα[3] and include fever, chemotactic effects on neutrophils, increased synthesis of acute

phase proteins and lymphocyte activation.[163] Both TNFα and IL-1 will be discussed in detail later.

Of the other interleukins, IL-6 is the most extensively studied.[5] It stimulates the release of acute phase proteins by the liver, under the stimulation of TNFα and IL-1. In humans IL-6 levels have been closely correlated with the severity and outcome of septic shock.[3,164] The administration of 0.03 μg/kg of *E. coli* LPS to horses resulted in an increase in serum IL-6 levels by one hour, peaking at 4 hours. The increase in serum IL-6 concentration was correlated with an increase in rectal temperature observed over the same time period.[165] Similarly the infusion of 0.5 μg/kg of *E. coli* LPS in neonatal foals resulted in the production of IL-6, although the time to peak concentration was significantly shorter. Interestingly, foals which had been colostrum deprived have a delayed and less pronounced IL-6 response.[166] Dose dependant production of IL-6 has also been described by other authors *in vivo*[167] and elevations in serum IL-6 concentrations have been reported to occur in over two thirds of horses with acute abdominal disease.[51] The magnitude of the increase correlated with mortality.[51] Furthermore, the presence of IL-6 has been correlated with detectable elevations in plasma LPS concentrations[51] and identified in foals with presumed septicemia.[168]

Interleukin-8 and granulocyte-colony stimulating factor (G-CSF) are other cytokines important in the activation and recruitment of leukocytes. IL-8 is released following stimulation by TNFα and IL-1 and is a potent agent in the recruitment and activation of neutrophils, while G-CSF is primarily responsible for regulating the proliferation, differentiation and activation of lymphocytes.[3]

Interleukin-10 is an important anti-inflammatory cytokine which is released as part of the compensatory anti-inflammatory response.[3,169] Interferon-γ is an important regulator of both the innate and adaptive immune response, although a specific role in equine endotoxemia has not been identified.[170] Both will be covered in more detail later.

Lipid Derived Mediators.

The majority of inflammatory mediators derived from lipid are the result of phospholipase A_2 hydrolysis of membrane phosphoglycerides resulting in the release of arachidonic acid and production of lyso-platelet activating factor (lyso-PAF). Arachidonic acid, a 20 carbon fatty acid, is subsequently metabolized by either 5-lipoxygenase or cyclooxygenase.[5] Lyso-PAF is further metabolized to the active inflammatory mediator platelet activating factor (PAF).[5]

The role of the cyclooxygenase pathway in the pro-inflammatory cascade observed in equine endotoxemia is well recognized. Cyclooxygenase metabolites of specific interest include thromboxane A_2, prostaglandin $F_{2\alpha}$, prostaglandin I_2 and prostaglandin E_2.[5,6] Many of the clinical signs of endotoxemia are mediated, at least in part, by these products.[171] During the initial stages of endotoxemia levels of thromboxane A_2 and prostaglandin $F_{2\alpha}$, both of which are potent vasoconstrictors, increase rapidly and are associated with the development of pulmonary hypertension, dyspnoea and hypoxemia.[5] Prostaglandin I_2 and prostaglandin E_2, both vasodilatory, are released later in the disease course. The release of prostaglandin I_2, in particular, is

associated with the development of systemic hypotension and the associated clinical signs including prolongation of capillary refill time. Central prostaglandin E_2 production is associated with the development of fever.[5]

The quantification of the products of the 5-lipoxygenase pathway, and thus determination of their importance to endotoxemia, remains elusive due to limitations in laboratory assays and the short half lives of the products.[5,171] However, four leukotrienes (LT), B_4, C_4, D_4 and E_4, which are produced by a variety of cells, have been studied.[172,173] Leukotriene B_4 is capable of inducing neutrophil adhesion, chemotaxis, enzyme release and oxygen free radical generation. The other three, collectively referred to as slow reacting substance of anaphylasis, are potent vasoconstrictors, bronchoconstrictors and are capable of increasing the permeability of post capillary venules.[172] The *in vitro* production of LTC_4 by equine neutrophils in response to LPS has been demonstrated.[174]

Platelet activating factor, also a lipid derived mediator, is important in the development of circulatory shock.[5] Serum and tissue PAF levels have been shown to increase in animal models of shock. Administration of PAF results in platelet aggregation, increases in microvascular permeability and hypotension.[175] Platelet activating factor has been shown to be a potent vasodilator in most tissues except in pulmonary, renal and coronary vasculature where it causes vasoconstriction.[3] *In vitro* PAF stimulates the production of thromboxane A_2 by equine peritoneal macrophages with a magnitude similar to LPS at 5 ng/ml[176] and PAF directly, not thromboxane A_2, is responsible for platelet aggregation.[177] The use of PAF-antagonists has suggested a role for PAF in LPS induced fever, tachycardia, hyperlactemia, leucopenia and decreased

gastrointestinal motility in horses.[178,179] The use of a PAF-antagonist has recently been shown in humans to decrease organ failure in patients with septic shock, emphasizing its potential importance in the condition, although no effect on mortality was observed.[180]

Coagulation/Fibrinolytic Factors.

The potential importance of abnormalities in coagulation or fibrinolysis is evidenced by the finding that over 90% horses in one study with colic attributable to gastrointestinal disease, a population with a high prevalence of endotoxemia, had disruption in at least one of the coagulation parameters (antithrombin III, thrombin clotting time, prothrombin time, fibrinogen degradation products, platelet count, plasma fibrinogen, partial thromboplastin time and soluble fibrin monomer) studied.[32] The number[181] or severity[33,36,182] of abnormalities in coagulation, and alterations in specific clotting factors including antithrombin III,[183] plasma factor VII[33] and protein C,[184] have been associated with worsening of prognosis in horses.

Prolongation of prothrombin, activated partial thromboplastin and whole blood recalcification times, and higher concentrations of fibrinogen, fibrinogen degradation products, plasminogen activator inhibitor, and alpha-2-antiplasmin have been identified in foals with presumed neonatal septicemia.[168] Elevations in prothrombin time and activated partial thromboplastin time have also occurred following experimental small intestinal strangulation obstruction in ponies.[185] Decreases in protein C antigen and antithrombin III activity have been identified in foals.[168]

A relationship between the presence of LPS and anomalies of coagulation in peritoneal fluid was established in a study of clinical cases of colic. The presence of LPS was associated with increases in the activity of tissue plasminogen activator activity, plasminogen, protein C, antithrombin III, α_2-antiplasmin and concentration of fibrin degradation products. These increases, with the exception of tissue plasminogen activator activity, were associated with increased mortality.[186] A recent report has demonstrated reduced mortality with supplementation of activated protein C in human sepsis.[187] Given the information above it is likely that a similar effect may be observed in the horse.

The History of Equine Experiments in Endotoxemia

The similarities between the clinical signs in horses with severe colitis and experimental endotoxemia was first observed in 1965.[188] Subsequent experiments in horses and ponies have continued to explore the similarities between LPS administration and a wide range of equine conditions including colic, colitis and metritis. An overview of some of the seminal *in vivo* experiments that have examined the administration of LPS and its effects in the equine, with particular relevance to the clinical presentation, is outlined below. More recently most of the work in equine endotoxemia has focused on the use of a wide range of *in vitro* models to identify various mediators implicated in the pathogenesis of the syndrome. Some of these experiments will be discussed in the next section with specific regard to the mediators investigated in this project.

In their original study Carroll *et al* (1965)[188] administered 25 μg/kg of *Aerobacter aerogenes* LPS to a single horse by intraperitoneal injection, and observed a rapid onset of clinical signs including collapse, nystagmus and colic within 1 hour, and death within 8 hours of injection. Several important comparisons to clinical disease were observed during this study. A profound leucopenia, especially neutropenia, rapidly developed followed by a left shift, and then a gradual increase towards normal values by eight hours. An initial elevation in packed cell volume and serum total solids protein was followed by a transient decrease in both and then a steady increase in both to above normal limits. Profound hypoglycemia just prior to death was also observed. Importantly the development of both diarrhea and severe colic in a previously clinically

normal animal was observed. Of equal importance was that Carroll *et al* (1965)[188] were the first to observe the extreme sensitivity of the horse to LPS when compared with other species, noting that the majority of mice who received 800 times the dose on bodyweight basis survived. Other authors have reported lower, but still differences of 100 fold, between the two species.[189,190] However, due to wide variation in the potency of LPS preparations direct comparison of studies is difficult.

Two studies comparing the effects of rapid and slow intravenous infusion of high doses of *E. coli* LPS in ponies under anesthesia then followed in 1970.[65,189] With administration of doses ranging from 100 to 300 μg/kg given over the period of one hour death was observed in all animals within eight hours. Seven of the nine animals died within two hours.[189] The effect of the infusion on several hemodynamic parameters, including mean arterial pressure, arterial pulse pressure, central venous pressure, left and right ventricular pressure and pulmonary artery pressure, was monitored. There was an initial and rapid decrease in mean arterial pressure and arterial pulse pressure, followed by a sharp transient increase then continual decrease until death occurred. Central venous pressure, right ventricular pressure and pulmonary artery pressure all increased concurrent with the decrease in mean arterial pressure then decreased back to control levels until death occurred. These findings were consistent with the development of systemic hypotension and transient pulmonary hypertension that had been observed in other species.[189]

The second of the two studies[65] compared the effects of a range of lower doses (32 to 200 μg/kg of *E. coli* LPS) given as an intravenous bolus. Ponies which survived 6

hours were allowed to recover from anesthesia. Two ponies which had received the highest doses died within 5 minutes of injection, and all of the ponies that received LPS died within 25 hours. The length of survival was directly related to the dose of LPS received. In animals that survived long enough, effects similar to those seen with the slow infusion on mean arterial pressure and central venous pressure were observed except that after a period 1.5 to 2 hours, a gradual increase in mean arterial pressure was observed until death occurred. This study also evaluated the effects of LPS on a number of hematological variables. Packed cell volume increased immediately following LPS administration and remained elevated until death. A profound neutropenia, worst at 60 to 90 minutes, occurred but resolved by 15 hours in the animals which survived long enough. Plasma glucose increased initially but then decreased after two hours.[65]

Burrows (1971)[191] published a third study in this series of experiments the following year where he evaluated the effects of a bolus dose of *E. coli* LPS in conscious ponies in a study similar to the second study above. At similar doses, similar effects on hemodynamic variables, blood glucose, white blood cell counts and packed cell volume were observed. Importantly the study noted the development of fever, tachycardia and a rebound neutrophilia with a pronounced left shift in animals which survived long enough. This has been reported in other species.[192] Interestingly, there was slightly better survival in this study, especially at higher doses suggestive that the combined cardiovascular depressive effects of anesthesia and endotoxemia may be important. However, overall the degree of similarity between the response in anesthetized and conscious animals validated the use of anesthetized animals for the study of endotoxemia.

Further work with 180 μg/kg of *E. coli* LPS in anesthetized ponies supported the hemodynamic responses described above although a pronounced decreased in central venous pressure was observed contrary to the above experiments.[190] This study also evaluated the effect of LPS challenge on parameters of oxygen delivery and found significant increases in arterial-venous oxygen difference, lactate and pyruvate and decreases in arterial pH and plasma bicarbonate concentration, suggestive of cellular hypoxia. A significant decrease in plasma sodium concentration was also observed but potassium and chloride concentrations were unaffected.[190] A much lower dose (10 μg/kg) administered to conscious animals produced similar results at 90 to 120 minutes. However, the period of acidosis occurred after a transient period of respiratory alkalosis and arterial pH returned to baseline values by 180 minutes.[193] The acidosis was also accompanied by a significant, almost immediate, hypoxemia, and, within 30 minutes, hyperlactemia, both of which occurred in the face of marked hyperventilation.[193] At this dose all ponies survived, however many experienced mild abdominal discomfort and all passed fluidy stool within 45 minutes of the infusion.

Further information on the effects of LPS on hemodynamic, hematological and plasma biochemistry parameters can be gleaned from a number of studies. Intravenous bolus of 200 μg/kg of *E. coli* LPS reproduced similar effects on plasma glucose, lactate, pH, packed cell volume, nucleated cell count, neutrophil count and morphology, and PaO_2 as had been previously reported.[194] Additionally, significant elevations in plasma insulin, cortisol, total and intestinal phosphatase, and creatinine phosphokinase brain

isoenzyme, were observed between four and six hours. No effect on plasma sodium concentration was observed in contrast to the above study.[194]

Using the lower doses of 10 or 15 μg/kg of *E. coli* LPS in horses Weld *et al* (1984)[195] reproduced similar effects on body temperature, hemogram, blood glucose, lactate and behavior as reported above but no significant hypotension was observed. In fact, at the higher of the two doses, a brief period of hypertension and a biphasic, then persistent tachycardia was observed. These findings strongly suggested a dose-dependent response to LPS. Differences in the response of horses to LPS, as compared with ponies which were widely used in the early experiments, cannot not be ruled out.

In an attempt to simulate clinical conditions more closely different routes of administration including intraperitoneal[69,188] and hepatic portal[196] routes have been studied. The original work by Carroll *et al* (1965),[188] described above, reported the use of intraperitoneal LPS and a subsequent report of intraperitoneal administration compared the effects of intraperitoneal, with intravenous administration of various doses of *E. coli* LPS.[69] The intravenous control group that received 150 μg/kg of LPS demonstrated similar effects to the above experiments. In comparison administration of 300 μg/kg of LPS by intraperitoneal injection resulted in a longer time to peak and duration of clinical signs and a higher mortality. Interestingly, the administration of 100 μg/kg of LPS q 1 hour intraperitoneal for 4 doses resulted in a similar, though more pronounced, delay in times to peak, and duration of clinical signs but mortality was less and similar to the intravenous LPS group. The effects of multiple injections on packed cell volume, neutrophilia and lymphocyte cell counts were of similar magnitude to the other two

groups but more prolonged in effect.[69] Overall, the author concluded that the use of intraperitoneal models may be more appropriate as the effects more closely resemble clinical disease.

The use of a hepatic portal infusion of 1 μg/kg/hr of *E. coli* LPS for 24 hours resulted in similar clinical signs as described above.[196] The onset of clinical signs including collapse, colic, delayed capillary refill time and sweating occurred rapidly, but had largely resolved by 2 hours, leaving the animals mildly sedated or depressed. Likewise the development of leucopenia and thrombocytopenia occurred quickly but values in both categories had begun to return towards baseline values by 8 hours despite continuation of the infusion. An increase in packed cell volume was observed similar to that reported in previous experiments but returned towards baseline values prior to discontinuation of the infusion at 24 hours. All of the animals survived the study, however all but one of the horses developed acute laminitis. Interestingly, the development of clinical signs consistent with laminitis was associated with a decrease, not increase, in hoof wall temperature. Hemostatic indices were also examined in this experiment. Consistent with a hypercoagulable state there was a transient decrease in prothrombin time and activated partial thromboplastin time, as well as an increase in fibrinogen and the development of fibrin degradation products,. The presence of a hypercoagulable state was transient and in two horses was followed by the development of a hypocoagulable state. One of the most important findings of this study was the apparent development of tolerance to LPS with constant exposure.[196]

Endotoxemia has also been reproduced in an experimental small intestinal strangulation obstruction model where the development of endotoxemia was correlated closely with mucosal degeneration, including villous atrophy.[54] Importantly this study demonstrated that the development of endotoxemia did not coincide with the release of the strangulated bowel, as is commonly thought, but was due to morphological changes within the bowel.

More recently *in vivo* research has focused on the effects of very low doses of LPS, with specific interest on its effects on the gastrointestinal tract.[197-200] The effect of 0.03 μg/kg of *E. coli* LPS on systemic and gastrointestinal hemodynamics has been studied by Clark *et al.*[197-199] The effects on systemic hemodynamics were far less pronounced than those seen in the higher dose experiments described above, with a mild, transient increase in heart rate, mean arterial blood pressure and respiratory rate and no effect on central venous pressure observed.[197,199] In the third of the studies at the same dose, tachycardia was observed but no effect on blood pressure, cardiac output and total peripheral resistance was seen.[198] Pulmonary artery pressures increased and there was a trend towards higher pulmonary vascular resistance, indicating that pulmonary hypertension can occur at lower doses of LPS without concurrent changes in systemic hemodynamics.[198] Even at such a low dose signs of cyanosis, colic and occasional diarrhea were observed.[199]

At the same dose there was a significant decrease in intestinal venous blood flow due to an increase in pre-capillary vascular resistance.[197] Decreased venous blood flow, due to vasodilatation and pooling of blood, had been previously reported at a higher dose

(200 μg/kg *E. coli* LPS).[201] The difference in mechanisms observed again points to a dose-dependent effect of LPS. The development of intestinal vasoconstriction appears to be an important compensatory mechanism during endotoxemia aimed at preserving systemic hemodynamics.[198] This may be self defeating as decreased blood flow leads to decreased mucosal blood flow, mucosal barrier dysfunction and absorption of further LPS.[197] The administration of 0.1 μg/kg of *E. coli* LPS resulted in a significant decrease in gastric, small colon and large colon motility as well as disruption to small intestinal motility although total stomach to anus transit time was not affected.[200] The effects of LPS on bowel motility appear to be mediated, in part at least by prostaglandins as the administration of prostaglandin E_2 resulted in disruption of motility in manner similar to that of LPS.[200]

Cytokines Studied

Tumor necrosis factor α, IL-1, IL-10 and IFN-γ represent a cross section of the important cytokines in the inflammatory response. They are not present at detectable levels in the serum of normal animals but are produced as part of the systemic inflammatory response observed following exposure to LPS. Tumor necrosis factor α and IL-1 are key pro-inflammatory mediators while IL-10 is the key anti-inflammatory mediator. Interferon-γ is an important immunoregulator with inmmunostimulatory and immunosuppressive effects on both the innate and adaptive immune responses.[170]

Tumor Necrosis Factor Alpha

Tumor necrosis factor α is part of a family of proteins produced by monocytes and macrophages. It is involved in regulation of apoptosis and cell proliferation, but it also has important pro-inflammatory actions and is involved in the recruitment and activation of a variety of cells, including neutrophils, macrophages and lymphocytes, resulting in production of pro-inflammatory cytokines and acute phase proteins.[3] It has been described as the principle mediator in endotoxic shock and its importance in sepsis has been demonstrated in a number of ways.[158] Firstly, injection of LPS into healthy volunteers results in rapidly detectable levels of TNFα.[202] Patients with sepsis have elevated levels of TNFα detectable within their blood and the degree of elevation is correlated with both severity of disease[159,160] and with outcome.[161,162]

Secondly, the administration of TNFα to animals and humans produces clinical and clinicopathological signs, including fever and hemodynamic and metabolic changes typical of sepsis.[203,204] Conversely, the administration of anti-TNFα antibodies decreases the severity of disease and mortality in laboratory animals.[205,206] Finally, disruption of the TNF gene results in decreased sensitivity to LPS in mice exposed to LPS but, emphasizing its importance as a mediator of the host defense increased susceptibility to infectious agents as has been reported with TLR4 and LBP.[207,208]

Numerous studies have evaluated the importance of TNFα in equine endotoxemia in both adults and neonates. The bolus injection of 5 μg/kg of *E. coli* LPS into foals produced a rapid (within 30 minutes), but transient, increase in serum TNF activity.[209] This preceded the peak alterations in heart rate, temperature and respiratory rate consistent with the notion that TNF is a early, intermediate cytokine rather than a direct effector cytokine.[209] A similar early, but transient peak in TNF activity was observed in adult horses with the infusion of 0.03 μg/kg/hr of *E. coli* LPS for four hours.[209] The transient nature of the response suggests that in the face of continual exposure TNF production is down regulated following an initial increase.[209] Bolus injection of 0.5 μg/kg *E. coli* LPS in neonatal foals produced a similar though slightly later peak in TNFα activity and undetectable levels by 24 hours.[210] Production of TNF in response to LPS stimulation in a dose-dependent manner has been described by other authors *in vivo*[167,211] while significant associations between serum TNF activity and the clinical signs of endotoxemia, including behavioural abnormalities, leucopenia, fever and tachycardia, have been made experimentally.[211]

In a large review of 289 horses with colic attributable to gastrointestinal disease elevated serum TNF activity was detected in 20% of horses.[212] Furthermore, in the same study TNF activity was linked to the condition with horses afflicted by strangulating small intestinal obstruction and proximal enteritis having significantly higher levels of TNF than all other groups except gastrointestinal rupture. Similarly, marked increases in TNF activity were positively correlated with increased mortality. A further association between LPS and TNF activity has been demonstrated recently with a correlation between the presence of LPS within the serum or peritoneal fluid, and TNF activity in horses with gastrointestinal disease. In both groups an increase in TNF activity was observed in the presence of LPS.[52] In contrast to the above study, serum TNF activity was not correlated with survival or disease type (except gastrointestinal rupture) however, peritoneal TNF activity was positively correlated with mortality and disease type. Higher concentrations of LPS and TNF were observed in peritoneal fluids when compared with serum.[52]

Increased serum TNF activity has also been documented in foals with presumed septicemia.[168] The ability of equine peritoneal macrophages to produce TNF has been demonstrated *in vitro*.[213,214]

Interleukin-1

Although equine IL-1 has been characterized for over 10 years[215] a specific role of IL-1 in equine endotoxemia has not been determined. However, there is substantial

evidence the IL-1 is an important mediator of septic shock in humans and other species and it is reasonable to expect that it is centrally involved in equine endotoxemia. IL-1 consists of two closely related proteins, IL-α and IL-1β, both of which have very similar properties and actions.[3] The action of IL-1 is similar to, though generally less pronounced than, TNFα and the two may act synergistically.[3,216,217] IL-1 is produced predominately by macrophages, but also by a variety of other cells, in direct response to LPS,[217,163] leukotrienes and TNF.[172,218,219] Actions of IL-1 include fever, chemotactic effects on neutrophils, increased synthesis of acute phase proteins and lymphocyte activation.[163] Many of the effects of IL-1 are likely mediated through its ability to induce phospholipase A_2 and cyclooxygenase-2.[217]

Similar to TNFα, serum concentrations of IL-1 have been shown to increase during endotoxemia[163] and have been correlated with severity of disease.[159] Treatment with a IL-1 receptor antagonist has resulted in improved survival in animals[220,221] but not humans.[222] Additionally, the administration of IL-1 reproduces some of the signs of endotoxemia, including fever, neutropenia, pulmonary edema and sequestration of granulocytes.[223]

Interleukin-10

Interleukin-10 has been described as the perfect anti-inflammatory cytokine.[3] It is not present in normal animals but is produced by a large variety of cells including mononuclear phagocytes, B and T lymphocytes and natural killer cells during sepsis.[224]

It is present in higher levels in sepsis when compared to controls, and during septic shock when compared with sepsis alone.[225]

Release of IL-10 is, in part at least, mediated by TNFα as part of a negative feedback loop of the cytokine response. *In vivo* evidence for this includes the production of less IL-10 and TNFα when mononuclear cell stimulation by LPS is accompanied by TNFα antibodies.[169] Evidence for the anti-inflammatory activity of IL-10 includes *in vitro* incubation of whole blood with LPS in the presence of anti-IL-10 antibodies resulting in significantly increased TNFα production.[225] Elsewhere IL-10 inhibition of the production of a variety of pro-inflammatory cytokines, including TNFα, IL-1α, IL-6, IL-8 and IL-12 in a number of *in vivo* studies has been demostrated.[224,226-229] Furthermore improved survival in IL-10 treated animals in endotoxemia and sepsis has been reported.[229] Deletion of IL-10 production genetically or with immunization results in increased mortality.[230,231]

Some authors have suggested that IL-10 may also have some effects at ameliorating the LPS induced changes in coagulation and fibrinolyis,[232] although other studies have failed to confirm this.[227] The effects of IL-10 on cell mediated immunity are unclear as both suppression[226] and stimulation[224] of cell mediated immunity have been demonstrated *in vivo* in experimental endotoxemia.

Only one study has being performed to date with IL-10 in horses. This study evaluated the use of recombinant human IL-10 in an *in vitro* model of LPS-stimulated equine peritoneal macrophages. A dose dependant suppressive effect on TNF, IL-6 and

prostaglandin E_2 production was observed. Tumor necrosis factor production was the most sensitive to the effects of IL-10.[233]

Interferon-gamma

The presence of IFN-γ has been known for nearly 30 years although its exact role in the inflammatory response is yet to be clarified.[170] Similarly, its role in equine endotoxemia has not been elucidated. However, it is an important regulator of both the innate and adaptive immune response. Interferon-γ is not present in the serum of normal animals but is produced by natural killer and T cells under certain stimuli, including infection and trauma. Production of IFN-γ is regulated primarily by IL-12, which up regulates production, and IL-10, which is a potent down regulator at physiological concentrations.[170]

With specific regard to the innate immune response IFN-γ is important in regulating the differentiation, stimulation and function of mononuclear phagocytes and enhancing the production of TNFα and IL-1.[170,234] Importantly, IFN-γ and TNFα have been shown to have synergistic effects in endotoxemia in a number of different ways.[235] Firstly, pretreatment of mice with anti-IFN-γ antibody prior to otherwise fatal LPS exposure significantly improves survival in a dose-dependent manner. In fact, pre-treatment with high doses of anti-IFNγ antibody was comparable to pretreatment with anti-TNFα antibodies. Secondly, mice treated with concurrent doses of TNFα and IFN-γ, which when given separately were non-lethal, all died. Thirdly, administration of anti-

IFN-γ antibodies blocked the lethality of TNFα in a dose-dependent manner. Finally, production of IL-6, an important secondary mediator, was synergistically enhanced by the concurrent administration of recombinant TNFα and IFN-γ.[235]

Current Treatments

In a recent review of 101 diplomates of American College of Veterinary Internal Medicine and American College of Veterinary Surgeons the most common treatments for endotoxemia were intravenous fluid therapy, non-steroidal anti-inflammatory drugs, broad spectrum antimicrobials, plasma and dimethyl sulfoxide (DMSO).[236] Correspondingly the initial focus of this review is on the use of those therapies.

Crystalloid Fluids

Given the profound effects of LPS on the cardiovascular system, correction of these deficits in an attempt to optimize oxygen delivery is a fundamentally important goal of therapy in endotoxemia.

Restoration of circulatory volume is most commonly achieved with the use of isotonic, polyionic electrolyte solutions such as lactated Ringers solution or Normosol-R®. These solutions are pH balanced and the predominant electrolytes are sodium, potassium and chloride. These solutions contain relatively high concentrations of sodium and chloride, in relation to plasma, which is beneficial during resuscitation or in animals with ongoing electrolyte loses, as derangements of these electrolytes are commonly seen.[42,190] There is a relatively low concentration of potassium in such fluids and potassium chloride (KCl) is commonly added to create a solution for long term use or maintenance. The addition of 20 – 40 mEq KCl per liter of fluids is generally safe during

the maintenance phase of fluid therapy. However, caution should be exercised to not exceed a maximum rate of 1 mEq KCl/kg/hour, as potentially fatal arrhythmias may occur.[237] In general, the addition of KCl to fluids during the acute resuscitative phase is not recommended to avoid an accidental excessive rate of administration.

In animals with clinically appreciable signs of dehydration, such as prolonged mucous membrane refill time, tacky gums or prolonged skin tent a minimum of 5%, and up to 10%, of the bodyweight (25 – 50 L for a 500 kg horse) should be provided rapidly to restore the circulatory volume. The rate of fluid administration in the adult horse is generally limited by the type of fluid administration set and catheter size used. Up to 60 ml/kg/hr (30 L/hr for a 500 kg horse) can be administered easily and safely[238] with the use of a 10 gauge jugular catheter and large bore administration set, such as arthroscopy tubing, or with a peristaltic pump. Caution should be exercised in severely hypoproteinemic animals as the combination of decreased colloid oncotic pressure and increased vascular permeability present in severe endotoxemia may predispose these animals to peripheral and, more importantly, pulmonary edema. Thus, prior or concurrent administration of colloids (discussed below) is beneficial in these animals.

Maintenance fluid requirements (40-60 ml/kg/day in adult horses) should be met, and ongoing losses replaced. Where possible, losses should be measured (i.e. gastric reflux) and this amount of fluid added to the maintenance requirement. Animals with severe diarrhea or large volumes of reflux may require in excess of 5L/hr of fluids. Monitoring of hematological variables such as packed cell volume (PCV) and total solids protein (TSP) is useful, but complicated by the multitude of factors which affect either

variable. For example serum protein, especially albumin, is commonly lost in severe gastrointestinal disease but the absolute TSP value may be within the normal range in a severely hypoproteinemic animal due to concurrent hemoconcentration. Similarly, PCV often remains elevated above normal reference values in severely stressed animals despite appropriate fluid resuscitation. The monitoring of plasma lactate, if available, provides a means of assessing tissue oxygenation. However, high levels may persist despite adequate fluid resuscitation due the presence of defects in cellular oxygen utilization that occur following endotoxemia or cellular hypoxia.[239] In foals, measurement of central venous pressure is easily performed in the hospital setting with indwelling jugular catheters and can allow more accurate titration of an animal's fluid needs.

The monitoring of multiple variables and the following of trends over time, taking into account all available information is recommended. A practical approach following restoration of the approximated deficit is to monitor the animal's clinical hydration status, urine output, losses (if possible) and hematological variables. Ongoing fluid therapy can then be tailored to effect. In general, if edema is not present, slightly excessive fluid administration is preferential to inadequate provision of requirements. If edema is present then colloid therapy should be considered following reassessment of renal and cardiovascular function.

Many animals with endotoxemia suffer from concurrent acidosis.[190] As described above, endotoxemia ultimately leads to cellular hypoxia, which is followed by a shift in cellular energy production to anaerobic metabolism. This results in production of lactic

acid and a metabolic acidosis. Correction of the acidosis may be achieved solely by the restoration of circulatory volume, and in most cases the use of alkalizing agents, such as sodium bicarbonate ($NaHCO_3$), is not required. Indiscriminant use of NaHCO3 should be avoided in foals with respiratory disease, in particular, as the increased carbon dioxide production which results from its use can actually worsen acidosis.

The administration of other electrolytes such as calcium and magnesium should be done on an "as needed" basis as determined by clinicopathological evaluation. Care should be taken when interpreting total serum calcium concentrations in hypoalbuminemic animals as they may be artificially decreased as a result of decreased protein binding. Approximated corrected total serum calcium can be obtained with the following equation;

Corrected total serum calcium (mg/dl) = Measured serum calcium – serum albumin + 3.5.[240] For example a patient with a measured serum calcium of 8.0 mg/dl and an albumin of 1.5 mg/dl will have a corrected total serum calcium of 8.0 – 1.5 + 3.5 = 10.0 mg/dl.

If supplementation is deemed necessary, the administration of 1 ml/kg of 23% calcium boroglucanate intravenously or enterally, followed by reevaluation is recommended. Excessive administration of calcium should be avoided, as calcium supplementation has been shown to increase mortality in animal studies of endotoxemia.[241,242] The significance of this finding has not been examined in the horse.

Indications for supplementation of magnesium are less clear and measured serum magnesium concentrations may not be a good indicator as extracellular fluid contains

only approximately 1% of the total body magnesium. However in animals with normal renal function supplementation of moderate amounts of magnesium is safe.[238] In the authors' hospital supplementation with magnesium is generally reserved for cases with idiopathic supraventricular tachycardia, ventricular arrhythmias or refractory hypocalcaemia. Magnesium can be supplemented using magnesium sulphate intravenously at rates up to 2 mg/kg/min, with the total dose not exceeding 50 mg/kg, or as magnesium oxide by mouth at 8 – 32 mg/kg/day.[238]

Colloidal Fluids

Colloids are large molecules which draw and retain fluid within the vascular space via osmotic forces. Colloids are useful during fluid therapy to ensure adequate colloid oncotic pressure is present, thus maintaining vascular volume and minimizing the development of tissue edema. Colloids can be either natural, such as plasma, or artificial, such as large branched sugars (Hetastarch®). The administration of colloids is indicated in animals with severe hypoproteinemia (total solids protein < 4.0 mg/dl), hypoalbuminemia (serum albumin < 1.5 mg/dl), and mild hypoproteinemia or hypoalbuminemia with evidence of peripheral or pulmonary edema.

Plasma is the most commonly used colloid in the horse[236] and is more extensively discussed below. It is readily available commercially, or it can be prepared using an appropriate donor and commercially available plasma collection sets. Care should be taken during administration as anaphylactic reactions, although uncommon, may occur.

Plasma can be dosed to effect by monitoring serum albumin or total solids protein. However, 10 ml/kg (i.e. 5 L for a 500 kg horse) is a recommended initial dose. The need for more plasma can be determined by repeating the measurement of total serum proteins as an estimate of colloidal oncotic pressure and by clinical assessment.

Hydroxyethyl starches, such as Hetastarch®, are an alternative to plasma and have some theoretical advantages due to their large average molecular size and low immunogenicity. They are an effective and convenient alternative to crystalloid fluids in the acute resuscitative phase and are largely retained within the vascular space, compared with crystalloids where 75% of the fluid administered is lost from the vascular space within 30 minutes of administration.[243,244] Furthermore, it has been shown that Hetastarch® decreases capillary leak in certain disease conditions[245] which may limit the development of tissue edema. Coagulopathies due to Hetastarch® have been reported in humans[246] and ponies[247] in a dose-dependent manner. Care should be exercised in patients with clinical or haemostatic evidence of a coagulopathy.[248] Administration of 10 ml/kg of Hetastarch® to normal ponies at 10 ml/kg/hr was described as safe, whereas doubling of the dose is associated with prolongation of partial thromboplastin time and depletion of von Willebrands factor:Ag and factor VIII:c.[247] In the authors' hospital Hetastarch® is most commonly used in animals with clinical endotoxemia at 5ml/kg. At this dose, it appears to both safe and effective, even in animals with mild coagulopathies.

Following administration of Hetastarch ®, estimation of colloidal pressure using total solids protein is inaccurate, as the Hetastarch® molecules are not being directly measured and TSP will be decreased due to the dilutional effects of Hetastarch®.

Colloidal osmotic pressure can be directly measured but this requires specific equipment and is not commonly performed. The serum half life of Hetastarch® in the horse is not known. Clinically the effect is largely lost after three days and repeated administration may be required in animals with a prolonged course of disease. Alternatively concurrent administration of plasma may prolong the clinical effect of Hetastarch®.

Hypertonic Fluids

Hypertonic saline may also be beneficial during the acute resuscitative phase and is readily available, cheap and easily stored and administered. Proposed mechanisms of action include the drawing of fluid into the vascular space due to increased serum osmolality, and improved cardiac contractility, either by a direct effect or mediated via the vagal nerve.[249] Hypertonic saline also exerts a vasodilatory effect on the peripheral circulation, especially the coronary blood flow, and may improve peripheral oxygen delivery to the tissues.[250] In dogs administration of 4 ml/kg of 7% hypertonic NaCl has been shown to result in rapid, transient restoration of cardiovascular performance, however the administration of large volumes of crystalloids (> 25 L for a 500 kg horse) must follow within 30 minutes as the effects of hypertonic saline are lost after this time.[250]

The rate of administration of hypertonic saline should not exceed 1 ml/kg/min as rate dependent, profound, transient hypotension has been reported in dogs.[250] It has also been proposed that care should be exercised in severely (8 – 10%) dehydrated animals, as

decreased renal function and increased mortality have been reported in laboratory animals with induced endotoxemia.[251] These findings have not been repeatable in sheep[252] and the significance in the horse is unclear. The authors' experience would suggest that that the use of hypertonic saline is safe, even in severely dehydrated animals, if followed by appropriate crystalloid and/or colloid therapy.

Non Steroidal Anti-Inflammatory Drugs (NSAIDs)

The role of lipid derived pro-inflammatory mediators, and in particular the products of the cyclooxygenase pathway, is well recognized in equine endotoxemia. Cyclooxygenase metabolites of specific interest include thromboxane A_2, prostaglandin $F_{2\alpha}$, prostaglandin I_2 and prostaglandin E_2.[5,6] Many of the clinical signs of endotoxemia are mediated, at least in part, via these products.[171] During the initial stages of endotoxemia levels of thromboxane A_2 and prostaglandin $F_{2\alpha}$, both of which are potent vasoconstrictors, increase rapidly and these increases are associated with the development of pulmonary hypertension, dyspnoea and hypoxemia.[5] Prostaglandin I_2 and prostaglandin E_2, both vasodilatory in action, are released later in the disease course. The release of prostaglandin I_2 is associated with the development of systemic hypotension while centrally prostaglandin E_2 is associated with the development of fever.[5] Platelet activating factor (PAF) is a by-product of phospholipase A_2 mediated release of arachidonic acid and is important in the development of endotoxic shock.[5] Platelet activating factor is a potent activator of coagulation and acts as a vasodilator in

most tissues, but causes vasoconstriction in the pulmonary, renal and coronary vasculature.[3]

The use of NSAIDs in the treatment of endotoxemia is rational as this class of drugs inhibits cyclooxygenase activity and the subsequent generation of the reactive metabolites described above. Evaluation of the effect of NSAIDs in equine endotoxemia and critical review of the literature is limited by the fact that most studies evaluating the use of NSAIDs involve treatment with the NSAID of interest prior to exposure to LPS. The relevance of this to the clinical case is unclear as significant LPS exposure has occurred by the time veterinary attention is received in most clinical cases of endotoxemia. The effect of NSAIDs under such conditions is less well described.

Recently the use of the NSAID ibuprofen has been shown in severe sepsis in humans to decrease production of arachidonic acid metabolites and to improve several clinical parameters, although no overall effect on mortality was observed.[253] Appropriate placebo controlled clinical studies in the horse are lacking, and it is unclear whether NSAIDs improve survival in the horse. However, substantial anecdotal evidence exists that survival rates for equine endotoxemia have improved significantly in association with the routine use of NSAIDs, specifically flunixin meglumine. Even if objective documentation of the ability to decrease mortality in equine endotoxemia is lacking, their ability to ameliorate many of the clinical signs associated with the disease and improve patient comfort justifies their use.

The most commonly used NSAID in equine endotoxemia is flunixin meglumine.[236] It has been extensively studied in many models of equine endotoxemia.

Concerns over toxicity and the ability of high doses of flunixin meglumine to mask the need for surgical intervention in colic led to investigation of lower doses.[254,255] Pretreatment with 0.25 mg/kg of flunixin meglumine has been shown in an equine model of endotoxemia to decrease the period of inappetence, block the development of colic and to blunt the rise in temperature that occurs after LPS administration.[254,255] Pretreatment with 1.1 mg/kg is more effective at ameliorating the tachycardia, tachypnea and fever[254,256] induced by LPS and has been shown to increase the time till death in fatal models of endotoxemia in the horse.[257,258] Flunixin meglumine at either dose does not prevent the development of leucopenia with initial exposure to LPS but 0.25 mg/kg does result in a less severe leucopenia with multiple LPS challenges.[255] Both doses have been shown to be equally effective at decreasing the development of hyperlactemia.[254] Only the higher dose has been shown to alter the development of hypoxemia and acidosis.[194,256] Furthermore, 1.1 mg/kg has been shown to delay the onset of hypotension and hyperdynamic shock and to decrease the magnitude of the decrease in blood flow to the brain, heart and large intestine.[194,201,257,258] The LPS induced production of thromboxane B_2 and prostaglandin $F_{1\alpha}$ is blocked effectively by 0.25 and 0.1 mg/kg respectively.[254]

With regards to evaluating horses with colic, the authors feel that the administration of 1.1 mg/kg of flunixin meglumine does not preclude accurate assessment of the need for surgical intervention. Other diagnostic procedures, such as examination per rectum, abdominocentesis, and the presence of borborygmi, as well as careful observation for subtle signs of pain, including depression and trembling, provide adequate information on which the decision for surgery can be based. With that in mind,

and given the dose-dependent effects of flunixin meglumine, the use of 1.1 mg/kg, q 12 hours may be superior to the "anti-endotoxemic dose" of 0.25 mg/kg, q 6 hours in decreasing the severity of disease. Alternatively, although not formally evaluated, the use of 0.5 mg/kg q 6 hours may offer some advantages to the standard lower dose, while alleviating any residual concerns over masking the indicators of surgical colic.

Other NSAIDs including phenylbutazone, ketoprofen, and eltenac are often used in horses with endotoxemia[236] and each has been studied in endotoxemia. Pretreatment with phenylbutazone, at 2 mg/kg intravenously, has been shown to delay the onset of clinical signs of endotoxemia. However, once developed, the clinical signs were unchanged compared with saline controls. Furthermore, in direct comparison, pretreatment with flunixin meglumine at 1 mg/kg intravenously almost completely ameliorated the clinical signs and blocked the rise in plasma lactate.[259] On the other hand phenylbutazone has been shown to block LPS induced decreased blood flow to the intestines more effectively than flunixin meglumine.[260] Given the significant difference in the two drugs ability to alter clinical signs and cardiovascular effects of endotoxemia the authors believe there is little indication for the use of phenylbutazone as an anti-endotoxemic treatment in the horse.

Ketoprofen has been proposed to have theoretical advantages over flunixin meglumine on the basis of partial inhibition of the leukotriene pathway and a higher therapeutic index.[261] An *in vitro* comparison of the two drugs found no difference in production of lipid derived mediators or TNFα following stimulation with LPS.[262] The authors feel that as its effectiveness is less well documented than flunixin meglumine,

and it appears to offer no clear-cut advantages that flunixin meglumine remains the NSAID of choice for the treatment of equine endotoxemia.

More recently eltenac has been evaluated with promising results. Pretreatment with 0.5 mg/kg intravenously resulted in amelioration of the development of fever and pulmonary hypertension observed following LPS exposure. This effect was likely due to the complete blocking of prostaglandin $F_{1\alpha}$ and thromboxane B_2 production seen.[263] Although no effect was seen on LPS-induced changes in heart rate, systemic blood pressure and white cell count eltenac did block the rise in endogenous cortisol and epinephrine which were used as markers of stress.[263] The authors believe that it is a potentially useful anti-endotoxic treatment worthy of further evaluation.

Antimicrobials

The use of antimicrobials in endotoxemia is controversial. In animals with defined infections, such as pneumonia and metritis, antimicrobial therapy is clearly indicated. Importantly, dying bacteria may liberate LPS and potentially worsen the clinical picture. However, rapidly reproducing bacteria liberate LPS and thus appropriate antimicrobial therapy may actually decrease overall LPS release. Antimicrobial associated release of LPS is dependent on the antimicrobial used. Antimicrobials effective against the bacterial cell wall, such as penicillins and cephalosporins, result in a greater release of LPS than those that affect microbial protein synthesis, such as the

aminoglycosides which result in minimal release of LPS.[264] The LPS liberating effects of penicillins are blocked by the concurrent use of an aminoglycoside.[264]

In horses with endotoxemia originating from gastrointestinal disease, a clear indication for antimicrobials is less apparent. Bacterial translocation from the gastrointestinal system is well recognized as an important secondary problem in human sepsis,[265] a disease condition which closely resembles endotoxemia. In the authors' opinion, broad spectrum antimicrobial therapy is indicated in severe cases of suspected endotoxemia.

In most cases the use of penicillin and gentamicin is effective and safe although care must be exercised in animals with renal disease. Doses of penicillin ranging from 20 000 – 40 000 IU/kg IV q 6 hours and of gentamicin of 6.6 mg/kg IV once daily are generally adequate. In mature animals with suspected renal disease enrofloxacin at 5.0 mg/kg IV, once daily is a viable alternative to gentamicin. Ceftiofur at 4 mg/kg IV or IM q 12 hours is another alternative although care should be exercised as substantial anecdotal evidence exists that the drug may be associated with a higher risk of antimicrobial associated colitis than other antimicrobials. The use of ceftiofur alone may also result in liberation of large amounts of LPS.[264]

Hyperimmune Plasma

Hyperimmune plasma is collected from horses that have been hyperimmunized with rough, or mutant, strains of gram-negative bacteria that lack the O-specific

polysaccharide chain. The loss of the O-specific polysaccharide chain exposes the toxic lipid A portion of the organism directly to the adaptive immune system and allows the generation of anti-lipid A antibodies. The rational 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.

Hyperimmune plasma has been studied in the horse in a number of different experiments with inconclusive results. Initial evaluation in both an experimental model in adult horses[266] and a clinical trial in foals[64] found no significant difference in any of the parameters studied when compared with normal plasma. However, subsequent evaluation in an experimental trial found a significant improvement in the behavioral abnormalities, including colic and anorexia, observed following LPS exposure in ponies.[267] These results were confirmed by a double blinded, placebo controlled trial in adult horses.[268] Furthermore, the later study found a significant decrease in mortality in the treatment group and a trend towards shortened recovery periods.[268] Most recently, the administration of hyperimmune plasma to foals with experimental endotoxemia has been associated with worsening of clinical signs and significant elevations in the key inflammatory mediators TNF and interleukin-6.[269] The reason for the inconsistency observed in the experimental and clinical trials is unclear. An age effect may be present as the two studies involving foals returned inconclusive or deleterious effects, while studies in adults tend towards a positive clinical effect. Whether this effect is real or whether other confounders are at play is unclear.

Based on the findings of the above studies hyperimmune plasma may be beneficial in the treatment of endotoxemia in adult horses but it should be avoided in foals as worsening of the clinical syndrome may occur. Although it remains unclear whether hyperimmune plasma is of benefit, it is important to recognize that fresh or fresh frozen plasma is an important therapeutic agent in treatment of endotoxemia and provides a readily available source of replacement protein. This is beneficial as the development of severe hypoproteinemia is common in animals with gastrointestinal disease as discussed above.

Dimethyl Sulfoxide (DMSO)

Dimethyl sulfoxide is extensively used in the treatment of equine endotoxemia,[236] however the drug has not been studied in either experimental or clinical equine endotoxemia. Free radical oxygen species (ROS) are highly reactive oxygen products that are commonly produced as a result of disruption of normal energy metabolism following periods of ischemia and reperfusion. Free ROS are extremely toxic to cells and DMSO has been shown to have potent ROS scavenging capacities and anti-inflammatory properties.[270-272] Additionally, DMSO reduces platelet aggregation.[271,273] It is likely these combined effects are beneficial in endotoxemia as ROS are released during endotoxemia[274] and hypercoagulation results in microthrombi formation and ischemia at the microvasculature level. Consequently it is reasonable to expect that DMSO may improve microvasculature circulation and oxygen delivery at the tissue level and,

combined with its anti-inflammatory properties, that it may be therapeutically advantageous in equine endotoxemia.

The pharmacokinetics of DMSO have been described in the horse[275] and it can be given enterally via nasogastric tube or intravenously. Intravenous administration of concentrations less than 20% is safe, while higher concentrations have been associated with intravascular hemolysis. Administration of doses up to 1.0 mg/kg as a 10 - 20% solution, intravenously or enterally, q 12 hours may be beneficial in the treatment of endotoxemia.

Polymyxin B

Polymyxin B is a cationic polypeptide with dose-dependent effects. At high doses the drug is a bactericidal antimicrobial with a predominately gram-negative spectrum of activity while at lower doses the drug binds the lipid A component of LPS.[276] In addition to binding free LPS, polymyxin B has been shown to disperse LPS aggregates.[276] The drug is potentially toxic with neurological signs and neuromuscular blockade most commonly reported. The development of signs of toxicity has been associated with doses of 18 000 unit/kg or greater,[277,278] while no toxic effects have been observed in healthy animals at doses of up to 10 000 unit/kg.[279]

The use of polymyxin B in the treatment of equine endotoxemia has been studied *ex vivo*[279] and *in vivo*[269] as has the effects of a polymyxin B-dextran 70 conjugate *in vivo*.[278] *Ex vivo* polymyxin B has a dose-dependent suppressive effect on TNF activity

and linear regression analysis indicates that 1 100 units/kg and 5 200 units/kg could be expected to suppress TNF activity by approximately 75% for 3 and 12 hours, respectively.[279] These results are supported by an *in vivo* study which examined the effects of treatment with 6 000 units/kg of polymyxin B prior to LPS exposure in which lower maximal TNF and IL-6 activities were observed.[269] Furthermore, polymyxin B attenuated the increases in respiratory rate and rectal temperature seen following LPS administration, suggesting that it may be useful in the treatment of equine endotoxemia. Similarly, the use of a polymyxin B-dextran 70 conjugate at 50 000 units/kg of polymyxin B resulted in undetectable levels of TNF and IL-6 following LPS exposure and significant lowering of heart rate, respiratory rate and temperature.[278] At this dose mild signs of toxicity were observed, however these were blocked by the concurrent administration of ketoprofen.[278] Polymyxin B relies on binding LPS and blocking its binding to mononuclear phagocytes. Thus its effect may be significantly reduced in clinical endotoxemia where LPS exposure has already occurred. The results of the above experiments indicate, however, that it may be a useful therapeutic modality under certain circumstances.

Pentoxifylline

Treatment of endotoxemia with pentoxifylline has become popular recently. Pentoxifylline is a xanthine derivative, which increases intracellular cAMP. It has been demonstrated to have beneficial effects, including improved cardiac output[280] and

increased oxygen delivery and tissue oxygen uptake[281,282] in a wide number of experimental models of sepsis in laboratory animals, to improve survival in septic human neonates.[283] Pentoxifylline inhibits, in a dose-dependent manner, TNF, IL-6 and tissue factor activity following *in vitro* LPS exposure of equine whole blood.[284] Important to the clinical setting, TNF production was inhibited by the addition of pentoxifylline after LPS exposure. This is consistent with the drug's ability to modulate the cellular response compared with other therapies (hyperimmune plasma, polymyxin B) which rely on preventing cellular binding of LPS.

Unfortunately, attempts to reproduce these effects *in vivo* in the horse have been disappointing. The use of pentoxifylline alone subsequent to LPS exposure in two separate studies resulted in some positive effects on clinical signs, with lower rectal temperatures and respiratory rates, but no effect on TNF or IL-6 activity or on the hematological parameters measured other than whole blood recalcification times.[285,286] The use of pentoxifylline in combination with flunixin meglumine was demonstrated to reduce the magnitude of the flunixin meglumine induced decrease in $PGF_{1\alpha}$ levels.[286] The significance of this finding is unclear.

A likely explanation for the inability to reproduce the results of the *in vitro* experiments is that the effects observed were dose-dependent and were seen at concentrations of 10 μg/ml or greater. In a study on the pharmacokinetics of pentoxifylline at doses similar to those used in both *in vivo* studies, peak serum concentrations following intravenous administration of only 3.5 μg/ml were achieved.[287] Similarly, in a model identical to that used by Barton *et al* (1997)[285] above,

administration of 7.5 mg/kg intravenously followed by an infusion of 3 mg/kg/hr resulted in a brief peak blood concentration of 9.6 μg/ml with a steady state concentration of 3.4 μg/kg.[288] Although pentoxifylline may be beneficial in endotoxemia and the potential usefulness of this has been demonstrated *in vitro* it is unlikely to have any significant effect when given intravenously at currently recommended doses. Furthermore the oral bioavailability of pentoxifylline has been demonstrated to be very poor and erratic[287] and no accurate recommendations can be made regarding its oral use on the basis of currently available information.

Heparin

The presence of a hypercoagulable state in horses with endotoxemia and severe gastrointestinal disease is well recognized and several studies have demonstrated prolongation of clotting times and depletion of multiple coagulation factors in clinical cases involving both adults and foals.[32,168] Alterations in specific clotting factors including antithrombin III,[183] plasma factor VII[33] and protein C[184] and the total number[181] or severity [36,182] of abnormalities in coagulation have been associated with worsening of prognosis.

Heparin is a potent anticoagulant and down regulator of the coagulation cascade. Although it may seem counterintuitive to administer an anticoagulant to an animal with prolongation of clotting times and bleeding tendencies, down regulation of an over activated system which is uncontrollably consuming clotting factors is a reasonable

therapeutic goal. Heparin exerts its effect predominantly via inhibition of thrombin, also called activated factor II, and by blocking the thrombin mediated positive feedback which occurs on factors V and VIII.[289] It also results in the release of tissue factor inhibitor by endothelial cells thereby decreasing factor VII activity, and blocks platelet aggregation.[290,291] The overall effect of heparin is down regulation of coagulation via inhibition of the extrinsic, intrinsic and common pathways, platelet aggregation and fibrinolysis.[292]

The pharmacology of heparin in the horse has been described.[292] Clearance occurs by a combination of saturable mechanisms and hepatic and renal, linear, first order metabolism and clearance. The combination of clearance mechanisms means that a linear dose effect is not observed and consequently a decreasing dosing regime is recommended. Based on titration of activated partial thromboplastin time and thrombin clotting time a dosing regime of a single loading dose of 150 U/kg subcutaneously (SQ), followed by 125 U/kg q 12 hours SQ for six doses and then 100 U/kg SQ q 12 hours until no longer required has been recommended.[292]

Future Treatment Considerations

Anti-TNF Antibodies

Tumor necrosis factor is the principle mediator in endotoxic shock and its importance in the pro-inflammatory cascade is well recognized.[158] Reasonably, it has been proposed that the use of monoclonal anti-TNF antibodies would decrease the severity of disease. The administration of anti-TNFα antibodies has been shown to decrease the severity of disease and mortality in laboratory animals.[205,206] Initial evaluation of murine monoclonal antibodies in miniature horses given LPS suggested a positive effect with decreased of serum TNF activity, improved clinical scores, heart rates and white cells counts.[293] However, these findings were not repeatable in subsequent study using rabbit monoclonal antibodies in adult horses.[294] Several reasons may exist for this disparity; firstly, a dose-dependent effect on TNF has been observed *in vitro*[294] and the dose used in the former study was nearly 20 fold higher. Secondly, although rabbit monoclonal antibodies have been demonstrated to be the most potent inhibitors of TNF activity in horses *in vitro*,[294] this effect may not be observed *in vivo*. Alternatively, the first study pretreated with the monoclonal antibodies whereas the second administered anti-TNF antibodies after LPS exposure. Finally miniature and normal horses may respond differently to LPS.

In a recent review of the use of anti-TNF therapy in humans with sepsis Reinhart and Karzai (2001)[295] concluded that meta-analysis of all randomized, controlled clinical

studies demonstrated a modest improvement in mortality of approximately 3%. However there is marked variation in the response of different subpopulations to anti-TNF therapy with patients in shock the most likely to benefit from therapy.[295] Given the modest results from human clinical trials and the inconsistency of equine studies it is unlikely that anti-TNF antibodies will be of significant benefit in equine endotoxemia.

Detergent

It has long been recognized that species which have a high density of pulmonary intravascular macrophages, such as horses and ruminants, have a dramatically increased sensitivity (100 to 1 000 fold) to LPS when compared with other species, such as primates and murine species.[296] Biosafe, intravenous detergents have been evaluated as a potential anti-endotoxemic therapy in these species with promising results.[296,297] Although the exact mechanism of action is unknown, several different mechanisms have been proposed. Firstly, detergents may physically interact with, and prevent the binding of LPS to lipopolysaccharide binding protein (LBP) or they may prevent the binding of the subsequent of the LPS/LBP complex to the primary cellular receptor, CD14. Alternatively, detergent has been shown to interfere with lipid metabolism and may directly inhibit arachidonic acid metabolism.[296]

In a recent study in horses, pretreatment with an intravenous detergent, tyloxapol, resulted in significantly lower pulmonary artery pressures and complete amelioration of the leucopenia and fever observed following LPS exposure.[296] Similar results have been

reported in sheep in which a greater than 90% suppression of TNF activity was also demonstrated.[297] Although further pharmacokinetic work needs to be performed to establish an appropriate dosing regime detergent may be a useful, safe and cheap treatment for endotoxemia.

Activated Protein C

In the twenty years preceding 2001 despite all the improvements in critical care including the continual development of new antimicrobials with greater spectrums of activity, no single treatment had been demonstrated to result in a significant reduction in overall mortality due to sepsis in humans.[187] However, in 2001 a novel therapeutic agent, recombinant activated protein C, was described.[187] Activated protein C is an important down regulator of the coagulation cascade that has antithrombotic, anti-inflammatory and profibrinolytic properties.

In a recent randomized, double blind, placebo controlled, multicenter trial recombinant human activated protein C was demonstrated to reduce the relative risk of mortality by nearly 20%.[187] This equates to one extra life saved per sixteen people entering the ICU. The results of this study confirm the importance of coagulopathies in sepsis and endotoxemia. Decreased levels of protein C have been associated with a worsening of prognosis in horses suggesting that a similar effect may be observed in the endotoxemic horse.[184] A potential role for recombinant activated protein C in equine

endotoxemia, especially neonatal critical care, has yet to be determined but warrants further investigation.

Insulin

More recently the regulation of blood glucose levels within normoglycemic levels in critically ill humans has been shown to result in a relative risk reduction for mortality of 32% when compared with a conventional permissive hyperglycemia approach.[298] Of potentially equal importance for veterinary medicine is that the intensive treatment group had a 46% reduction in secondary septicemia, decreased long term requirement for antimicrobials and shortened ICU stays.[298] Almost 100% of patients in the intensive treatment group required insulin therapy, however with the use of controlled delivery pumps and regular blood glucose monitoring, maintenance of normoglycemia with a constant rate infusion of insulin is an achievable goal in equine critical care, especially in neonates. The systemic manifestations of endotoxemia in horses and sepsis in humans are very similar and the maintenance of normoglycemia with the use of insulin in endotoxemic horses may contribute to a significant reduction in the morbidity and mortality associated with endotoxemia.

Literature Review – Summary

Endotoxemia is a very important disease condition that occurs secondary to numerous primary disease conditions that are common encountered in the equine. It is a major contributor to morbidity and mortality in both adults and foals and results in large financial losses to the equine industry on an annual basis.

Clinical and clinicopathological signs of endotoxemia have been reproduced experimentally and closely resemble the clinical syndrome. The cellular pathways that lead to the wide array of alterations in homeostasis that are observed following exposure to LPS are beginning to become well described and the current concept is that the syndrome is the result of over activation of the innate immune response. The importance of various serum proteins and cellular receptors has been demonstrated and the interaction of LPS with LBP, CD14, TLR and MD-2 is generally considered vital for cellular activation, although other receptors may be important under specific conditions.

Several mediators of the innate immune response, including TNFα and IL-1, have been identified as important factors in endotoxemia. Others such as IL-10 and IFN-γ may play an important role regulation of the immune response and warrant further investigation.

Despite an increased understanding of the pathophysiological processes involved in endotoxemia current therapy is largely aimed at supportive care and inhibition of specific metabolites of arachidonic acid metabolism. While this is undoubtedly

beneficial in affected animals, the mortality rate of animals with suspected endotoxemia remains high and continued research into potentially more effective therapies is required.

Historically research into the treatment of equine endotoxemia has focused on blocking the interaction of LPS with effector cells or inhibition of specific cellular metabolites. While such therapies may be useful under certain condition the efficacy is often limited in the clinical setting. Evaluation of treatments which affect the complex cellular signaling process may be more rewarding in the clinical setting.

PGG-Glucan

The use of yeast cell wall products as immunostimulants dates back to the early 1960's.[299] Recently patients with concurrent fungal infections were found to have consistently low or negligible levels of circulating LPS despite confirmed gram-negative sepsis.[300] This observation lead to the suggestion that fungal products may enhance LPS clearance and the evaluation of fungal products, such as β-Glucans, as adjunctive treatments to antimicrobial therapy in sepsis.

PGG-Glucan is a soluble β-(1,6)-branched β-(1-3)-linked glucan derived from the cell wall of the yeast *Saccharomyces cerevisiae*. It is an novel immunomodulator that has been shown to decrease the severity of infection and mortality associated with sepsis in a variety of animal models.[301-305] It also decreased the post operative infection rate in human high-risk surgical patients.[306,307] It has been widely studied in laboratory animals as an immunostimulant, both alone and in conjunction with antimicrobials.

Although the exact mechanism of action is not known, PGG-Glucan increases neutrophil oxidative burst activity and decreases the severity of bacterially induced leucopenia.[301] PGG-Glucan alone does not induce an active oxidative burst but enhances the response to other activators in dose-dependent manner.[308] The ability of PGG-Glucan to enhance microbicidal activity appears to be mediated via activation of a NF-κB-like nuclear transcription factor following binding to a specific glycosphingolipid receptor.[308] The affinity of PGG-Glucan for the glycosphingolipid receptor is higher than other, less branched, β-Glucans.[305] PGG-Glucan

appears to be fairly non-specific as it is effective against both gram-positive and gram-negative organisms.[305]

The use of PGG-Glucan at doses ranging from 0.25 mg/kg to 4.0 mg/kg resulted in increased clearance of the organism in an intraperitoneal model of *Staphylococcus aureus* infection.[301] When combined with ampicillin, microbial clearance was greater than with either treatment alone.[301] This effect has also been observed in other studies along with decreased mortality in fatal models.[304,305] PGG-Glucan achieved this effect without significantly increasing the white cell count or inducing TNFα or IL-1β production. The *S. aureus* induced decrease in white blood cells was lower in the PGG-Glucan treatment group. A threshold effect was observed in this study with regards to dose. Doses of 0.5 mg/kg and higher were equally effective.[301] This is in contrast to a guinea pig model of staphylococcal wound infection, and a mouse model of peritonitis, where 1.0 mg/kg of PGG-Glucan was found to be the most effective dose, with efficacy reduced at higher as well as lower doses.[303,305]

PGG-Glucan has been evaluated in a number of human studies. The inoculation of healthy human volunteers with doses ranging from 20 – 140 mg/person resulted in a dose dependant increase in absolute neutrophil count and a dose and time dependent effect on unstimulated leukocyte chemiluminescence, which was used as a marked for cellular microbicidal activities.[302] The dose-dependent effect was observed up to 60 mg/person with maximal effect seen at both 60 mg and 140 mg. Maximal chemiluminescence was observed at twenty four hours and increased microbicidal activity of leukocytes versus *S. aureus* was seen 3 to 6 hours after 60 mg.[302]

The wealth of evidence in laboratory animals lead to PGG-Glucan being evaluated in Phase I and II human trials. In a single center, randomized, double-blind, placebo controlled phase I/II trial, doses ranging from 0.05 to 2.25 mg/kg were safe and well tolerated. A mild, transient increase in leukocyte numbers was observed.[307] A significant effect of treatment was observed with fewer infectious complications, shorter intensive care unit stay and decreased intravenous antimicrobial requirement when compared with the control group.[307] This study was extended into a multicenter, randomized, double blind, placebo controlled phase II trial evaluating a range of doses from 0.1 mg/kg to 2.0 mg/kg. Although doses as high as 2.25 mg/kg had previously been reported to be well tolerated[307] a high end dose of 2.0 mg/kg was used as some healthy volunteers had reported minor adverse reactions with 2.25 mg/kg.[306] In this study, a trend towards lower rates of infection amongst the treatment groups of doses 0.5 mg/kg, or greater, was observed. This did not reach statistical significance with the exception of a subset of serious life threatening infections where treatment with PGG-Glucan was beneficial.[306] A phase III trial was initiated. The results were inconclusive and further evaluation of PGG-Glucan as a prophylactic immunostimulant in humans was aborted as it was considered unlikely to get Federal Drug Administration approval for human use. (pers. comm. Gary Ostroff, Alpha-Beta Technology Inc)

Although PGG-Glucan does not induce production of TNFα or IL-1β[301,308] it is capable of modulating the cytokine production observed following *in vivo* stimulation of mononuclear cells with LPS or staphylococcal enterotoxin in mice.[309] Following pretreatment with PGG-Glucan, the overall cytokine patterns of isolated monocytes and lymphocytes reflected suppressed production of pro-inflammatory mediators and

enhanced production of IFN-γ. Specifically, following pre-treatment with PGG-Glucan, production of TNFα in response to LPS stimulation was attenuated. Conversely IFN-γ production was enhanced in lymphocytes isolated from PGG-Glucan treated mice subsequently exposed to staphylococcal enterotoxin B or toxic shock syndrome toxin 1 when compared with untreated mice.[309]

This ability to effect cytokine production in a murine model of endotoxemia lead to the investigation of PGG-Glucan as a potential anti-endotoxic therapy in this study.

Cover Sheet

PGG-Glucan Paper

The following manuscript contains the experimental component of the thesis, including material and methods, and results.

The article is primarily the work of Dr. Ben Sykes and the experiments were performed by Dr Ben Sykes in their entirety with the exception of the following; The TNFα assays in part 1 of the experiment were outsourced and performed commercially by the University of Georgia as approved by Dr. Martin Furr (Committee chairman). Similarly, the real time polymerase chain reaction was performed at the University of Florida, in part by Dr. Ben Sykes and in part by Dr Steeve Giguère's laboratory staff.

Manuscript preparation was primarily by Dr. Ben Sykes with structural and editorial input by Dr. Martin Furr. Dr. Steeve Giguère contributed with processing of the data from part 2 of the experiment and with input on the wording of the materials and methods sections relating to real time polymerase chain reaction. Statistical analysis was performed by Dr. Martin Furr and Dr. Ben Sykes.

TITLE PAGE

PGG-Glucan, a novel immunomodulator, alters TNFα response to endotoxin in horses.

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ABSTRACT

Objective – To determine the effect of PGG-Glucan on the *in vitro* and *ex vivo* cytokine response to endotoxin in the horse. In addition, the ability of PGG-Glucan to stimulate tumor necrosis factor alpha production *in vitro* was investigated.

Animals – Experiment 1; 6 adult horses. Experiment 2; 12 adult horses.

Procedure – Part 1: Whole blood was collected, aliquoted and incubated *in vitro* in four groups: saline control, endotoxin (LPS) control (100 ng/ml), PGG-Glucan (0.1, 1.0, 10 and 100 μg/ml) and LPS (100 ng/ml) plus PGG-Glucan (0.1, 1.0, 10 and 100 μg/ml). Supernatants were collected at 0, 6 and 12 hours and assayed for tumor necrosis factor α (TNFα) activity. Part 2: Horses received either PGG-Glucan (1 mg/kg) or an equal volume of isotonic saline (0.9% NaCl) IV over 15 minutes. Twenty four hours later blood was collected and mononuclear cells isolated for cell culture. Cells were treated with LPS (100 ng/ml) and RNA extractions were performed at 0, 6, 12, 24 and 48 hours. Relative mRNA expression of tumor necrosis factor α, interleukin-1β (IL-1β), interleukin-10 (IL-10) and interferon-γ (IFN-γ) was determined by reverse transcription and real time polymerase chain reaction.

Results – The production of TNFα *in vitro* by equine whole blood was attenuated by co-incubation with PGG-Glucan in a dose-dependent manner. This effect was observed

following incubation with saline, and in response to endotoxin challenge. *In vivo* PGG-Glucan administration had no effect upon the *in vitro* cytokine mRNA expression of TNFα, IL-1β, IL-10 or IFN-γ by peripheral blood mononuclear cells.

Conclusions and Clinical Relevance – PGG-Glucan is unlikely to have a significant effect upon clinical endotoxemia based on the modest changes observed on *in vitro* TNFα production following co-incubation with PGG-Glucan. However, alternative mechanisms of action of PGG-Glucan, other than modulation of cytokine production, that have been described were not evaluated in this study. Whether PGG-Glucan would be of benefit in equine endotoxemia via an alternative mechanism of action was not determined.

TEXT OF ARTICLE

Introduction

Endotoxemia results in major losses to the equine industry on an annual basis.[2] It is a major contributor to mortality and morbidity in both neonates and adults. Clinical and clinicopathological signs of endotoxemia have been evaluated as prognostic indicators in horses with severe gastrointestinal disease and correlated with increased mortality.[17,24,40,48,49]

The complex cellular response that occurs following exposure to endotoxin is becoming more completely described. Over 150 separate mediators have been identified although the importance of various key pro-inflammatory cytokines such as tumor necrosis factor α (TNFα) and interleukin-1β (IL-1β) has been known for some time.[3] The current concept is that the clinical manifestation of endotoxemia results from dysregulation of the innate immune response with unchecked up-regulation of the pro-inflammatory response, normally a vital protective mechanism.[4] As such, down regulators of the immune response may play an important role in controlling the severity of disease. Interleukin-10 (IL-10) has been described as the perfect anti-inflammatory cytokine.[3] Administration has been found to improve mortality in experimental models of sepsis.[229] Interferon-γ (IFN-γ) is an important immunoregulator with inmmunostimulatory and immunosuppressive effects on both the innate and adaptive immune responses.[170] Interferon-γ has also been shown to be an important regulator of endotoxin (LPS) induced lethality.[235]

Currently veterinary therapy for endotoxemia is largely aimed at the removal of the initiating cause, supportive care and inhibition of metabolites of the arachidonic acid cascade.[236] The use of substances that bind free LPS and prevent its interaction with cells of the mononuclear phagocyte system and subsequent activation of the innate immune response is a theoretically desirable goal. While some products such as polymyxin B, have shown promise,[269,278,279] clinical studies are lacking and investigation of other products, such as hyperimmune plasma, have been inconclusive.[64,266-269] Alternatively, modulation of the cellular response and down regulation of the innate immune response may be advantageous. Pentoxifylline, a methylxanthine derivative, has been shown to modulate the cytokine response in human sepsis.[283] However, despite promising *in vitro* results in the horse[284] evaluation of the drug *in vivo* has been largely unrewarding.[285,286] Furthermore, bioavailability of pentoxifylline in the horse is poor and highly unpredictable which greatly limits its clinical usefulness.[287]

PGG-Glucan is a soluble β-(1,6)-branched β-(1-3)-linked glucan derived from the cell wall of the yeast *Saccharomyces cerevisiae*. It is a novel immunomodulator that has been shown to decrease the severity of infection and mortality associated with sepsis in a variety of animal models[301-305] and to decrease the post operative infection rate in human high-risk surgical patients.[306,307] Furthermore, pretreatment with PGG-Glucan *in vivo* has been shown to attenuate TNFα production by murine lymphocytes and monocytes following *ex vivo* stimulation with LPS or staphylococcal enterotoxin.[309] Lymphocytes isolated from the PGG-Glucan treated mice and subsequently exposed to staphylococcal enterotoxin B or toxic shock syndrome toxin 1 had

enhanced interferon-γ production when compared with lymphocytes isolated from untreated mice.[309]

We hypothesized that PGG-Glucan would modulate the cytokine response of equine whole blood and isolated peripheral blood mononuclear cells following exposure to LPS and tested this hypothesis in *in vitro* and *ex vivo* experiments. The null hypotheses were:

Part 1 a). – Tumor necrosis factor α activity in whole blood following incubation with LPS *in vitro* with PGG-Glucan is equal to tumor necrosis factor α activity in whole blood following incubation with LPS *in vitro* without PGG-Glucan. b). - Tumor necrosis factor α activity in whole blood following incubation with saline *in vitro* with PGG-Glucan is equal to tumor necrosis factor α activity in whole blood following incubation with saline *in vitro* without PGG-Glucan.

Part 2 - *Ex vivo* mononuclear cell cytokine mRNA expression induced by LPS following treatment with PGG-Glucan is equal to *ex vivo* mononuclear cell cytokine mRNA expression induced by LPS following treatment with saline.

The alternative hypotheses were:

Part 1 – a). – Tumor necrosis factor α activity in whole blood following incubation with LPS *in vitro* with PGG-Glucan does not equal tumor necrosis factor α activity in whole blood following incubation with LPS *in vitro* without PGG-Glucan. b). - Tumor necrosis factor α activity in whole blood following incubation with saline *in vitro* with PGG-Glucan does not equal tumor necrosis factor α activity in whole blood following incubation with saline *in vitro* without PGG-Glucan.

Part 2 - *Ex vivo* mononuclear cell cytokine mRNA expression induced by LPS following treatment with PGG-Glucan does not equal *ex vivo* mononuclear cell cytokine mRNA expression induced by LPS following treatment with saline.

Materials and Methods

Experiment 1- *In vitro* endotoxin and PGG-Glucan challenge

Horses – Six healthy male castrate adult light breed horses were used. Their general health was determined the day prior to the experiment by clinical examination and routine hematological examination, including total white cell count, differential and determination of fibrinogen concentration. The hair over the left jugular vein was clipped and aseptically prepared and fifty milliliters of blood collected into lithium heparin tubes[a]T by venipuncture.

Experimental design – Nine hundred microliters of blood from each horse was aliquoted to 12 incubation plates such that 4 treatments could be analyzed at 3 time points. The 4 groups were: a) untreated saline control, b) positive (+) endotoxin (LPS) control, c) PGG-Glucan only treated, and d) LPS and PGG-Glucan treated. The 3 time points were 0, 6, and 12 hours of culture. The untreated saline control samples were placed into 1 ½ ml multiwell plates[b] with 100 µl of 0.9 % sodium chloride (NaCl). The positive LPS control samples had 100 µl of 1 µg/ml *Escherichia coli* 055:B5 LPS[c] in 0.9% NaCl added to a final LPS concentration of 100 ng/ml. Positive PGG-Glucan[d] controls were created by the addition of 100 µl of PGG-Glucan in 0.9% NaCl resulting in final concentrations of 0.1, 1.0, 10 and 100 µg/ml in the media. The fourth group consisted of 100 µl of 0.9% NaCl with LPS and PGG-Glucan added to achieve final concentrations of 100 ng/ml, and 0.1, 1.0, 10 and 100 µg/ml, respectively. The plates were then incubated at 37^0C and 5% CO_2 in a humidified incubator.

Tumor necrosis factor alpha assay - The 0, 6 and 12 hours samples from each treatment group were resuspended by gentle agitation, transferred to tubes and centrifuged at 400 g for 10 min. Time 0 samples were resuspended immediately following addition of the appropriate treatment (NaCl, LPS, PGG-Glucan, or LPS and PGG-Glucan) and centrifuged without delay. The supernatant was collected and stored at –70^{o}C until assayed for TNFα activity.

Tumor necrosis factor α activity was determined by an *in vitro* cytotoxic bioassay using the murine tumor cell line WEHI 164 clone 13, as previously described.[211,310] The assay is extremely sensitive and can detect TNFα concentrations as low as 0.1 pg/ml.[310] In brief, the WEHI cells were suspended in media containing fetal calf serum and actinomycin and placed in 96 microwell plates. Following incubation, diluted samples and positive controls, using human recombinant TNFα, were added to the cells and incubated for 18 hours. The cells were then incubated with the tetrazolium salt MTT for 4 hours and cell survival determined using colorimetrical analysis and a standard curve.

Statistical analysis – Results were entered into a standard desktop PC using commercial statistical analysis software.[e] After data entry was validated, data were summarized and descriptive statistics produced. The effect of PGG-Glucan treatment, LPS stimulation and time on TNFα activity was analyzed using ANOVA for repeated measures[e] after confirmation of appropriate statistical assumptions for this test by examining a plot of residuals. Data not meeting the assumptions of the ANOVA were transformed as

necessary. Post hoc pairwise comparisons between groups were made using Tukey-Kramer's test to identify specific changes. Results were expressed as mean ± SD, and significance was set at $P < 0.05$.

Experiment 2 - *Ex vivo* endotoxin challenge

Horses – Twelve healthy male castrate adult light breed horses were examined and determined to be healthy by clinical examination, routine hematological and serum biochemistry examination. The horses were age matched into pairs and randomly assigned to either treatment or control groups. On day 1 the hair over the left jugular vein was clipped and aseptically prepared. A 14-gauge teflon catheter[f] was aseptically inserted and secured. For the treatment group a PGG-Glucan solution of 1 mg/ml was diluted in 500 ml 0.9% sodium chloride at a final dose at 1 mg/kg. Control horses received 500 ml plus 1 ml/kg 0.9% sodium chloride. The PGG-Glucan or saline was infused over 15 minutes. Rectal temperature, heart rate, borborygmi and respiratory rate were determined prior to, and immediately following the infusion. Twenty four hours later a thorough clinical examination was performed, the right jugular vein clipped, the skin aseptically prepared and 80 ml of blood collected by venipuncture into lithium heparin tubes.[a]

Cell culture preparation – The heparinized blood was centrifuged at 600 g for 10 minutes at 20^{o}C and the buffy coat removed and suspended in RPMI 1640 incomplete medium[g] to a final volume of 12 ml. Two ml of the cell suspension was placed on top of 4 ml of

ficoll-hypaque in sterile 15 ml tubes at room temperature and centrifuged at 350 g for 30 minutes at 20°C. This was repeated to use the entire sample. The mononuclear cells were recovered from the ficoll-hypaque interface by gentle suction, resuspended in incomplete RPMI 1640 medium and centrifuged at 600 g for 5 minutes. Cells were washed in incomplete RPMI 1640 medium three times then resuspended in complete RPMI 1640 medium[h] containing l-glutamine, Hepes Buffer, 10% heat inactivated fetal bovine serum and penicillin (50 IU/ml)/streptomycin (50 μg/ml).

Cell counts were performed by hemocytometer and the cell suspensions diluted in complete RPMI 1640 medium to a concentration of 1 x 10^6 cells/900 μl. Nine hundred μl of the solution was placed into the well of a 1 ½ ml multiwell plate for determination of cytokine production at times 0, 6, 12, 24 and 48 hours. One hundred μl of complete RPMI 1640 was added to the wells for time 0 while 100 μl of complete RPMI 1640 medium plus 1 μg/ml of *E. coli* 055:B5 LPS[c] was placed into the wells for the remaining times giving final concentrations of 1 x 10^6/ml and 100 ng/ml for cells and LPS, respectively. Samples were set up in duplicate for each animal and incubated at 37^0C and 5% CO_2 in a humidified incubator.

RNA isolation, DNAse treatment of RNA samples, and cDNA synthesis - At each time point the cells were resuspended with gentle agitation, collected and transferred to separate tubes then centrifuged at 300 g for 5 minutes at 20°C. The supernatant was removed and RNA extraction performed on the remaining cell pellet using the RNeasy total RNA isolation kit.[i] RNA concentration was measured by optical density at 260 nm.

All RNA samples were treated with amplification grade DNAse I[j] to remove any traces of genomic DNA. Briefly, 1 U of DNAse I and 1 μl of 10 X DNAse I reaction buffer were mixed with 1μg of total RNA in a 10 μl reaction. The mixture was incubated for 10 min at room temperature and then inactivated by adding 1 μl of 25 mM EDTA and heating at 65 °C for 10 min.

cDNA was synthesized with the Advantage RT-for-PCR Kit[k] by using the protocol of the manufacturer. Briefly, 1 μg of total RNA was mixed with 1 μl of oligo $(dt)_{18}$ primer (20 μM) and heated at 70°C for 2 min. After cooling to room temperature the following reagents were added: 4 μl of 5 X reaction buffer (containing 250 mM Tris-HCl [pH 8.3], 375 mM KCl, and 15 mM $MgCl_2$), 1 μl of deoxynucleoside triphosphates (dNTP, 10 mM each), 0.5 μl of RNase inhibitor (40 U/μl) and 1 μl of Moloney murine leukemia virus reverse transcriptase (200 U/μl). The mixture was incubated at 42°C for 1 h, heated at 94°C for 5 min, diluted to a final volume of 100 μl, and stored at -70°C until used for PCR analysis.

Real time PCR – Gene specific primers and internal oligonucleotide probes for equine TNFα, IL-1β, IL-10, IFN-γ and glyceraldehyde-3-phosphate dehydrogenase (G3PDH) have been described previously.[311] The internal probes were labeled at the 5' end with the reporter dye 6-carboxyfluoresceine, and at the 3' end with the quencher dye 6-carboxytetramethyl-rhodamine. Amplification of 2 μl of cDNA was performed in a 25 μl PCR reaction containing 900 nM of each primer, 250 nM of TaqMan probe and 12 μl of

TaqMan Universal PCR Mastermix.[1] Amplification and detection were performed using the ABI Prism 7700 Sequence Detection System[m] with initial incubation steps at 50°C for 2 min and 95°C for 10 min followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. Serial dilutions of cDNA from 24 h Concanavallin A-stimulated equine blood mononuclear cells were used to generate a standard curve for the genes of interest. Each sample was assayed in triplicate and the mean value was used for comparison. Samples without cDNA were included in the amplification reactions to determine background fluorescence and check for contamination. To account for variation in the amount and quality of starting material, all the results were normalized to G3PDH expression. The threshold cycle values for each gene were compared to its respective standard curve to generate a relative transcript level.

Statistical analysis - Expression of mRNA for each cytokine relative to G3PDH was calculated for each sample and expressed as a ratio for statistical analysis. Results were entered into a desk-top PC, using a commercial statistical analysis software package.[e] Data was summarized and descriptive statistics performed. The effect of treatment and time on cytokine mRNA expression was analyzed using ANOVA of repeated measures,[e] after confirmation of appropriate statistical assumptions for this test by examining a plot of residuals. Data not meeting the assumptions of the ANOVA were transformed as necessary. Post hoc pairwise comparisons between groups were made using Tukey-Kramer's test to identify specific changes. Results were expressed as mean ± SD, and significance was set at $P < 0.05$.

Animal Care – The use of animals in both studies was reviewed and approved by the Virginia Polytechnic Institute Animal Care and Use Committee.

Results

Experiment 1 – *In vitro* endotoxin and PGG-Glucan challenge

Results of experiment 1 are shown in table 1 and summarized in Figures 2 and 3. Data met the assumptions of ANOVA and analysis was performed without transformation. Following treatment with endotoxin, production of TNFα increased by 6 hours, remaining elevated at 12 hours ($P<0.0001$). After 6 hours a difference in TNFα bioactivity was observed between the endotoxin control (3490.0 +/- 5366.0 pg/ml) and 0.1 μg/ml PGG-Glucan treated groups (1651.0 +/- 1777.9 pg/ml) ($P=0.047$). This effect was not observed at the 12 hour time point, and there were no other differences noted between various dosage groups. (See Figure 2)

An effect of co-incubation with PGG-Glucan on whole blood TNFα bioactivity in the absence of endotoxin was seen at 6 hours ($P=0.002$)(See Figure 3). The highest TNFα concentration was found in the control group which had not received PGG-Glucan. Tumor necrosis factor α bioactivity was significantly less than the control (21.2 +/- 34.0 pg/ml) in the cells treated with 10 and 100 μg/ml PGG-Glucan (4.8 ± 0.2 pg/ml ($P=0.036$), 12 ± 1.7 pg/ml ($P=0.011$) respectively). (See Figure 3)

Experiment 2- *Ex vivo* endotoxin challenge

No effects on clinical parameters were noted following treatment with PGG-Glucan (data not shown). The results for relative cytokine mRNA determination are summarized in table 2. Data for all the cytokines examined failed to meet the assumptions for the

ANOVA, demonstrating an increasing variance as absolute values increased. Log transformation of the data corrected the distribution and permitted further analysis.

Compared to time 0 an increase in TNFα mRNA expression was noted following incubation of cells with endotoxin, with or without PGG-Glucan ($P<0.0001$). This was greatest at 6 hours, and decreased thereafter with the exception of the 24 hour saline control. At 24 hours of incubation, the PGG-Glucan treated group had a TNFα mRNA expression that was about 33% of the non-treated control. This did not achieve statistical significance, however, presumably due to the large standard error found in the control group.

As seen with TNFα mRNA, the production of IL-1β mRNA increased following endotoxin challenge, with or without PGG-Glucan, peaked at 6 hours and significantly ($P<0.05$) decreased until the final assay at 24 hours. At the 24 hour time period, values for IL-1β mRNA had not yet decreased to baseline in either the control or PGG-Glucan treated groups ($P=0.005$ and 0.01, respectively).

Following stimulation with LPS, production of interleukin-10 mRNA increased at 6 hours, with or without PGG-Glucan. At the 12 and 24 hour time periods, values for IL-10 mRNA in the control group had decreased to baseline, but the values for the PGG-Glucan treated cells remained greater than baseline ($P=0.01$). Although there was no statistical difference between the control and treated groups for IL-10 mRNA at 24 hours, the PGG-Glucan treated group remained 3.5 times the baseline value.

Interferon-γ mRNA values increased following LPS exposure, with or without PGG-Glucan, peaking at 24 hours and remaining increased at 48 hours of culture in the

control group. Peak production of IFN-γ mRNA in the PGG-Glucan group was not observed until 48 hours. No significant differences in response were noted between the control and PGG-Glucan treated cells at any time point.

Discussion

PGG-Glucan decreases the severity of infection and mortality in a variety of animal models[301-305] and has been shown to modulate the *in vivo* cytokine response of mice exposed to LPS and staphylococcal enterotoxin.[309] The exact mechanism of action of PGG-Glucan is unknown although it did not alter pro-inflammatory cytokine production in humans and laboratory animals.[301,308] It may, however cause a mild, transient increase in white cell count in both humans and laboratory animals and has been shown to attenuate the leucopenia that develops following exposure to *Staphylococcus aureus.*[302,304,305,307] Whether a similar effect occurs in the horse is unknown since hematology was not repeated after treatment with PGG-Glucan in this study. PGG-Glucan has also been shown to increase neutrophil oxidative burst activity and to enhance microbicidal activity. This appears to be mediated through activation of a NF-κB-like nuclear transcription factor following binding to a specific glycosphingolipid receptor.[308] This effect occurs indirectly by enhancing the leukocyte response to other activators.[308] Combined with its potential leukoproliferative properties it might, at least in part, explain it's antimicrobial effect in other species.[301]

PGG-Glucan, alone, did not induce production of TNFα by equine whole blood *in vitro*. The lower TNFα activity in the PGG-Glucan treated controls at 6 hours *in vitro* when compared with the activity in saline treated controls, and similarly the absence of a significant difference between the PGG-Glucan treatment groups and saline controls in baseline values for the cytokines studied in the *ex vivo* part of the experiment indicates that induction of cytokine mRNA expression and transcription does not occur following treatment with PGG-Glucan. However, a transient effect that had gone by 24 hours would not have been detected and cannot

be ruled out. These findings are consistent with an *in vitro* study using human whole blood where increased TNFα, IL1α or IL-1β levels were not observed following co-incubation of LPS and PGG-Glucan, at 1 and 100 μg/ml of PGG-Glucan for 3 and 24 hours respectively.[308]

A dose-dependent suppressive effect on TNFα production was observed *in vitro*, both in the presence and absence of LPS. In the presence of LPS the most significant effect was seen at the lowest dose tested (0.1 μg/ml) while in the absence of LPS the reverse was true, with the greatest effect seen at higher doses (10 and 100 μg/ml). The dose-dependent nature of the response is consistent with *in vivo* experiments in humans and other species where the maximal effect is observed at doses of 0.5 to 1.0 mg/kg.[301,303,305,306] Above this range a threshold effect is generally observed[301,306] although decreased efficacy at both higher and lower doses has been reported.[303,305] The different nature of the dose-dependent response observed between LPS stimulation and saline control is interesting and may reflect an interaction between both the concentration of PGG-Glucan and the intensity of the stimulus for cytokine production. The cause of the increase in TNFα production at 6 hours in all groups, including the saline control, in the absence of LPS stimulation is unclear although it may be the result of mild cellular stimulation during collection and pipetting.

PGG-Glucan failed to alter the production of mRNA for the cytokines studied *ex vivo*. This is consistent with a study in mice where *in vivo* challenge with *S. aureus* had no effect on TNFα or IL-1β levels despite pretreatment with multiple doses of PGG-Glucan.[301] However, several effects may have been masked by the high level of variance present. Although statistical significance was not achieved, TNFα mRNA levels were nearly 7.5 times higher at 24 hours compared with baseline in the saline control group whereas the 24 hour values in the PGG-

Glucan treatment group were less than 70% of the baseline values. This suggests a suppressive effect of PGG-Glucan on TNFα production. In a similar experiment in mice pretreatment with PGG-Glucan resulted in a decrease of nearly 50% in the production of TNFα at 8 hours following exposure to LPS *ex vivo*. However, at 24 hours the converse was observed with TNFα production significantly higher in PGG-Glucan treated mice in comparison with controls, suggestive of a prolonged low level release of the cytokine.[309] This effect was not observed in our study. No effect of PGG-Glucan on Il-1β mRNA production was observed in our study.

Interleukin-10 mRNA levels remained over 3.5 times above baseline levels at 12 and 24 hours in the PGG-Glucan treatment group whereas the saline control group had returned to sub-baseline levels. No statistically significant effect was seen, although this may have been masked by the high level of variance in the study and if real, the ability of PGG-Glucan to suppress TNFα production and enhance IL-10 production would be consistent with a potent anti-inflammatory effect which is likely to be of benefit in equine endotoxemia.

No effect on IFN-γ mRNA production following pretreatment with PGG-Glucan and *ex vivo* stimulation with *E. coli* LPS was observed in mice.[309] However, following stimulation with staphylococcal enterotoxin B or toxic shock syndrome toxin 1, significantly enhanced production of IFN-γ was observed at 15 and 24 hours, but not at 48 hours.[309] In our study peak production of IFN-γ mRNA following LPS stimulation appeared delayed in the cells from PGG-Glucan treated horses when compared with the saline treated controls but statistical significance was not achieved.

The cause of the high level of variance present in the *ex vivo* study is unclear however several reasons may exist. These include inter-animal variability in the response to PGG-Glucan

or variability between the responses to LPS of peripheral blood mononuclear cells isolated from different animals. Contributors to such inter-animal variability generally include age, sex, breed and prior disease history. An attempt was made to minimize the effect of several variables by age matching horses prior to randomization into treatment and control groups and with the use of only male castrates. However, due to the limited availability of horses, breed could not be controlled for and the disease history of most horses used in the study was unknown.

Alternatively, experimental variability may have been responsible for the high level of variance seen. Multiple steps were required for processing of the samples and any variation that may have occurred early in the experiment would likely have been amplified. There is some evidence to suggest that a major contributor to the variance occurred prior to the RT-PCR. This is based on the observation that there was a large amount of variability in the amount of RNA present in the samples following RNA extraction. This may have occurred prior to RNA extraction as a result of inconsistencies in experimental technique, such as pipetting, variability in the survival of different cell cultures or it may have occurred during RNA extraction. Although the manufacture's protocol for RNA extraction was adhered to closely technical difficulties with the extraction, predominately difficulty in achieving consistent centrifuge speeds, were encountered.

Aside from the high level of variance, several other reasons may exist for failure of the *ex vivo* experiment to demonstrate an effect on peripheral blood mononuclear cell cytokine mRNA production despite the TNFα suppressive effects observed *in vitro*. As discussed above the effects of PGG-Glucan in other species are dose-dependent.[301,303,305,306] The pharmacokinetics of PGG-Glucan in the horse are not known although, based on this study, the administration of

PGG-Glucan to horses at a dose of 1 mg/kg is well tolerated and does not appear to be associated with any deleterious effects clinically. This is consistent with dose studies in humans where doses up to 2 mg/kg are well tolerated.[306,307] As no effect of PGG-Glucan treatment at 1.0 mg/kg was observed it is possible that a dose-dependent response exists and that higher or lower doses may be effective.

Timing of the *ex vivo* experiment may have been a factor. The timing of peripheral blood mononuclear cell isolation was identical to the murine study described above.[309] Furthermore maximal chemiluminescence, a marker of microcidal potential, is observed at twenty four hours in unstimulated leukocytes collected from healthy human volunteers pretreated with PGG-Glucan.[302] Thus the choice of pretreatment 24 hours before mononuclear cell isolation was probably appropriate. A different time dependent effect in the horse is possible. The administration of multiple doses has been used in experimental studies evaluating the use of PGG-Glucan as an anti-infective including a Phase II clinical trial[301,305,306] and may have been more likely to identify an effect. However, administration of a single dose has been shown to be effective at preventing wound infection with *Staphylococcal* sp.[303] and to modulate the cytokine response of mice to LPS and staphylococcal enterotoxin.[309]

In summary, PGG-Glucan produced a dose-dependent effect on TNFα production *in vitro*, both in the presence and absence of LPS. A similar effect was not observed *ex vivo* although the high degree of unexplained experimental variance present may have confounded results. Alternative mechanisms of action of PGG-Glucan, including enhanced microcidal activity and leukoproliferative effects, reported in other species that

may be of benefit in equine endotoxemia were not examined. Based on this study, a single dose of PGG-Glucan given 24 hours before a horse is exposed to LPS is unlikely to be beneficial in the treatment of clinical endotoxemia in the horse.

FOOTNOTES

[a] Vacutainer, Becton Dickinson VACUTAINER systems, Franklin Lakes, NJ.

[b] 48 Well cell culture cluster, Corning Inc, Corning, NY.

[c] Escherichia coli 055:B5 LPS, Sigma-Aldritch Corp, St. Louis, MO

[d] PGG-Glucan, Biopolymer engineering Inc, Eagan, MN.

[e] SAS statistical software, SAS Institute Inc, Cary, NC.

[f] Abbocath-T 14G, Abbott laboratories Inc, Abbott Park, IL.

[g] RPMI 1640 incomplete medium, Sigma-Aldrich Inc, St Louis, MO.

[h] RPMI 1640 complete medium, Sigma-Aldrich Inc, St Louis, MO.

[i] RNeasy total RNA isolation kit, Qiagen Inc, Valencia, CA.

[j] DNAse I , Gibco BRL, Rockville, MD,

[k] Advantage RT-for-PCR Kit , Clontech, Palo Alto, CA,

[l] TaqMan Universal PCR Mastermix, Applied Biosystems Foster City, CA.

[m] ABI Prism 7700 Sequence Detection System, Applied Biosystems, Foster City, CA.

General Summary

Equine endotoxemia is a serious disease in horses and a major cause of morbidity and mortality in both adults and foals. Treatment for equine endotoxemia at present in largely aimed at supportive care and, although several new treatments offer promise, finding an effective treatment should remain a primary research goal in equine medicine.

A continually growing understanding of the processes that leads to the development of endotoxemia following exposure to LPS is critical in developing new therapies. Given the complicated process involved, it is unlikely that any specific single anti-mediator therapy will be effective and modulation of the cellular response may be a more rewarding approach. As such, the investigation of drugs, such as PGG-Glucan, which modulate the overall cellular response is important.

In this study PGG-Glucan was shown to have only modest effects on pro-inflammatory cytokine production. Based on the findings of this study further investigation of PGG-Glucan as a modulator of cytokine production in equine endotoxemia is unwarranted. Further evaluation of PGG-Glucan investigating alternative mechanisms of action may yield more favorable results. Future directions for research in equine endotoxemia should include the use of anti-inflammatory cytokines, such as IL-10, or agents such as LBP and CD14 which exhibit dose-dependent effects on cellular activation and LPS clearance.

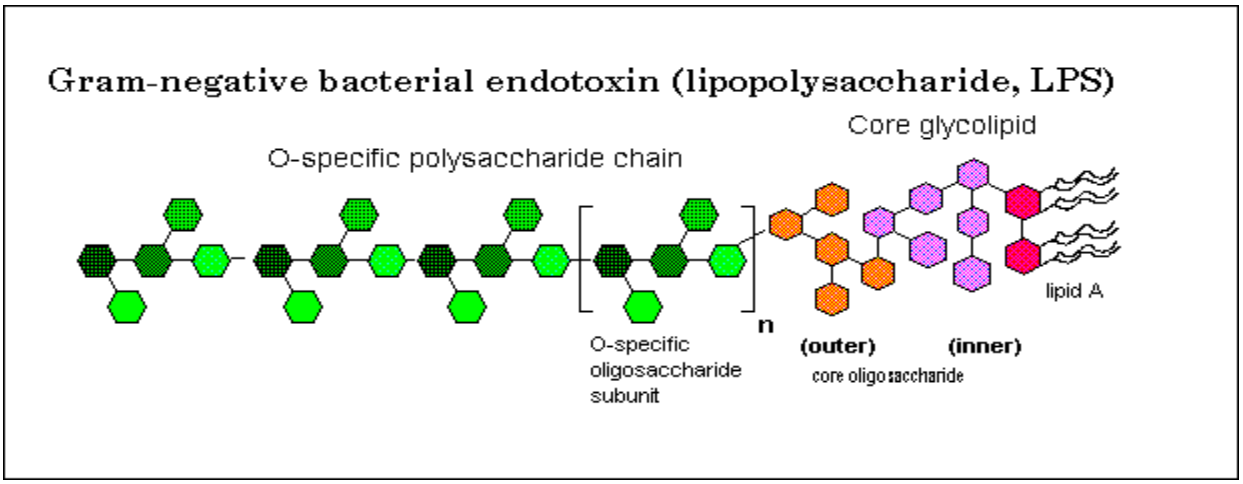

Figure 1. Structure of Lipopolysaccharide

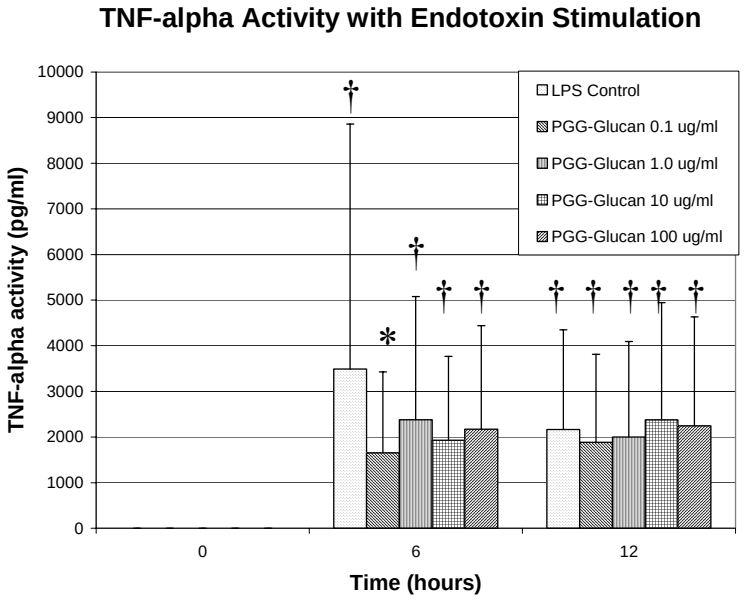

Figure 2. *In vitro* effect of PGG-Glucan on tumor necrosis factor α (TNFα) bioactivity (pg/ml) without LPS stimulation. Equine whole blood from 6 horses was incubated in the presence of LPS in the presence of four different concentrations of PGG-Glucan. Tumor necrosis factor α production was determined by an *in vitro* cytotoxic bioassay using the murine tumor cell line WEHI 164 clone 13. Values reported represent mean ± SD values for times 0, 6 and 12 hours. * Represents statistically significant different value ($P<0.05$) from LPS control for same time point. † Represents statistically significant different ($P<0.05$) from time zero for same treatment group.

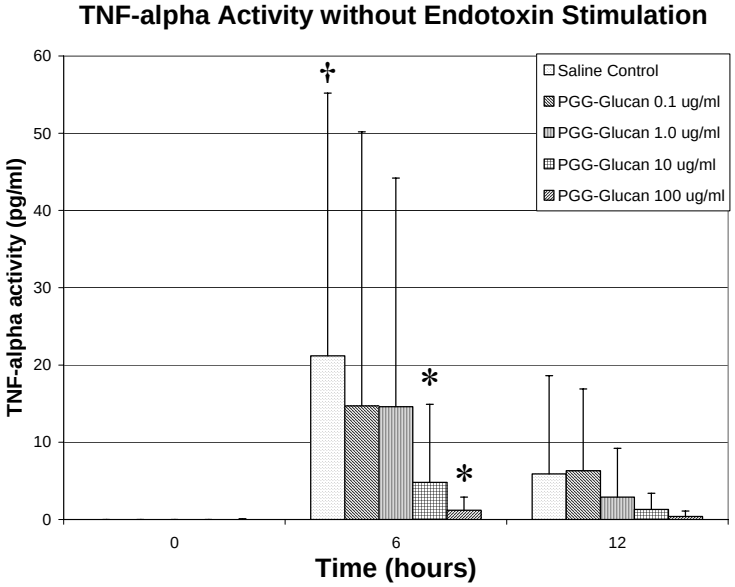

Figure 3. *In vitro e*ffect of PGG-Glucan on tumor necrosis factor α (TNFα) bioactivity (pg/ml) after stimulation by LPS. Equine whole blood from 6 horses was incubated in the presence of four different concentrations of PGG-Glucan. Tumor necrosis factor α production was determined by an *in vitro* cytotoxic bioassay using the murine tumor cell line WEHI 164 clone 13. Values reported represent mean ± SD values for times 0, 6 and 12 hours. * Represents statistically significant different value ($P<0.05$) from saline control for same time point. † Represents statistically significant different ($P<0.05$) from time zero for same treatment group.

Table 1. Mean ± SD values for tumor necrosis factor α (pg/ml) in *in vitro* whole blood cultures from 6 horses. Four treatment groups (untreated saline control, positive (+) LPS control, PGG-Glucan only treated, and LPS and PGG-Glucan treated) were evaluated at 3 time points (0, 6, and 12 hours) of culture. Tumor necrosis factor α production was determined by an *in vitro* cytotoxic bioassay using the murine tumor cell line WEHI 164 clone 13.

	O Hours	6 Hours	12 Hours
Saline control	0.0 ± 0.0	21.2 ± 34.0†	5.9 ± 12.7
LPS control 100 ng/ml	0.0 ± 0.0	3490.2 ± 5366.1†	2162.5 ± 2184.2†
PGG-Glucan 0.1 µl/ml	0.2 ±0.4	14.8 ± 35.5	6.3 ± 10.6
PGG-Glucan 1.0 µl/ml	0.0 ± 0.0	14.5 ± 29.5	2.9 ± 6.3
PGG-Glucan 10 µl/ml	0.0 ± 0.0	4.8 ± 10.2*	1.3 ± 2.1
PGG-Glucan 100 µl/ml	0.0 ± 0.1	1.2 ± 1.7*	0.4 ± 0.7
PGG-Glucan 0.1 µl/ml + LPS 100 ng/ml	0.1 ± 0.2	1650.9 ± 1777.8*	1884.5 ± 1931.2†
PGG-Glucan 1.0 µl/ml + LPS 100 ng/ml	0.0 ± 0.0	2380.0 ± 2699.1†	2001.5 ± 2091.8†
PGG-Glucan 10 µl/ml + LPS 100 ng/ml	0.0 ± 0.1	1928.0 ± 1838.4†	2377.5 ± 2567.7†
PGG-Glucan 100 µl/ml + LPS 100 ng/ml	0.0 ± 0.0	2169.4 ± 2272.8†	2245.3 ± 2387.7†

* Represents statistically significant different value ($P<0.05$) from respective control (saline or LPS) for same time point. † Represents statistically significant different ($P<0.05$) from time zero for same treatment group.

Table 2. Mean ± SD ratios for relative mRNA expression of tumor necrosis factor α, interleukin 1β, interleukin-10 and interferon-γ in peripheral blood mononuclear cell cultures following *in vitro* exposure to LPS 24 hours after treatment with either PGG-Glucan (n = 6 horses) or 0.9% NaCl (n = 6 horses).

		0 Hours	6 Hours	12 Hours	24 Hours	48 hours
TNFα	PGG-Glucan	5.76 +/- 15.66	11.93 +/- 10.30†	5.82 +/- 4.18	3.95 +/- 4.53†	
	Control	1.66 +/- 2.07	11.32 +/- 8.79†	7.07 +/- 4.47†	12.33 +/-27.51†	
IL-1β	PGG-Glucan	0.04 +/- 0.04	0.4 +/- 0.26†	0.20 +/- 0.12†	0.18 +/- 0.10†	
	Control	0.05 +/- 0.04	0.4 +/- 0.21†	0.29 +/- 0.16†	0.26 +/- 0.39†	
IL-10	PGG-Glucan	0.63 +/- 1.27	4.71 +/- 3.06†	2.70 +/- 2.85†	2.26 +/- 2.15†	
	Control	2.68 +/-8.30	3.04 +/- 3.10†	2.30 +/- 1.67	1.83 +/- 1.85	
IFNγ	PGG-Glucan	0.08 +/- 0.09		1.10 +/- 0.96†	1.38 +/- 1.92†	6.79 +/- 12.79†
	Control	0.21 +/- 0.36		1.18 +/- 1.40†	5.03 +/- 11.36†	3.81 +/- 5.22†

† Represents statistically significant different ($P<0.05$) from time zero for same treatment group. No significant difference was found between control and PGG-Glucan treated cells for any cytokine at any time point.

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VITA

Benjamin William Sykes was born October 8th, 1974 in Shepparton, Victoria, Australia to Bill and Sally Sykes. He was raised on a mixed enterprise farm in Benalla in north east Victoria and later in Darwin in northern Australia.

Ben graduated from Melbourne Church of England Grammar School in 1991. Following twelve months leave he commenced a veterinary degree at Murdoch University in Perth, Western Australia. He received a bachelor of science and a bachelor of veterinary medicine and surgery in 1995 and 1997, respectively, and was awarded the Australian College of Veterinary Surgeons prize in veterinary epidemiology. After graduation he moved to Sydney, New South Wales and completed an internship in equine medicine and surgery at Randwick Equine Center.

Ben then returned to Murdoch University as a registrar in equine medicine and surgery a position which he held for eighteen months prior to spending five months as the resident veterinarian at Woodlands stud, Denman, New South Wales. In January 2001, Ben commenced a residency in equine medicine at the Marion duPont Scott Equine Medicine Center, Leesburg Virginia and a Masters in Veterinary Science at Virginia Polytechnic Institute. After completion of his residency Ben has accepted a position as a lecturer in equine medicine at the University of Helsinki, Finland.

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[i] Vacutainer, Becton Dickinson VACUTAINER systems, Franklin Lakes, NJ.
[ii] 48 Well cell culture cluster, Corning Inc, Corning, NY.
[iii] Escherichia coli 055:B5 LPS, Sigma-Aldritch Corp, St. Louis, MO
[iv] PGG-Glucan, Biopolymer engineering Inc, Eagan, MN.
[v] SAS statistical software, SAS Institute Inc, Cary, NC.

[vi] Abbocath-T 14G, Abbott laboratories Inc, Abbott Park, IL.
[vii] RPMI 1640 incomplete medium, Sigma-Aldrich Inc, St Louis, MO.
[viii] RPMI 1640 complete medium, Sigma-Aldrich Inc, St Louis, MO.
[ix] RNeasy total RNA isolation kit, Qiagen Inc, Valencia, CA.
[x] DNAse I, Gibco BRL, Rockville, MD,
[xi] Advantage RT-for-PCR Kit, Clontech, Palo Alto, CA,
[xii] TaqMan Universal PCR Mastermix, Applied Biosystems Foster City, CA.
[xiii] ABI Prism 7700 Sequence Detection System, Applied Biosystems, Foster City, CA.