RESPONSE OF EQUINE MONOCYTES TO LIPOPOLYSACCHARIDE FROM ENTERIC AND NON-ENTERIC BACTERIA: EFFICACY AND RECEPTOR SPECIFICITY

by

KATHARINA LUISE LOHMANN

(Under the Direction of James N. Moore)

ABSTRACT

Endotoxin (lipopolysaccharide, LPS) activates inflammatory cells by interacting with a cellular receptor complex consisting of cluster differentiation antigen 14 (CD14), Toll-like receptor 4 (TLR4) and MD-2. LPS antagonists are structurally atypical LPS compounds that do not activate inflammatory cells and in fact inhibit cellular activation by endotoxin. LPS antagonists may be of therapeutic value and offer a tool for investigation of ligand-receptor interactions and species-specific differences in the response to different LPS compounds.

This report describes the results of an investigation of the biological activities of three structurally atypical LPS compounds in equine and human cells. LPS from *Rhodobacter sphaeroides* (RsLPS), *Rhizobium galegae* (*R. galegae*) and *Rhizobium Sin-1* (*R. Sin-1*) stimulated tumor necrosis factor α (TNFα) production in equine monocytes while they inhibited the response to enteric LPS in a human monocyte cell line, Mono Mac 6. Using transfection experiments, it was determined that RsLPS stimulated nuclear factor κB (NF-κB) activation in equine cells but not in human cells, and that stimulation of equine cells occurred independent of CD14. It was further determined that the TLR4/MD-2 complex determined the equine-specific response to RsLPS. Using transfection experiments and binding assays, it was determined that
LPS from *R. galegae* and *R. Sin-1* activated equine cells via CD14, TLR4 and MD-2 and that these compounds competed with enteric LPS for binding to equine monocytes. Contrary to their effect on TNFα production, LPS from *R. galegae* and *R. Sin-1* also stimulated NF-κB activation in cells expressing human receptor proteins, thereby putting into question a competitive mechanism of antagonism for these compounds. In an additional study, binding assays were used to estimate the number of binding sites for LPS on Mono Mac 6 cells and the affinity of LPS binding to these cells. Comparison to cell stimulation assays suggested the presence of spare receptors. Competition experiments further suggested an allosteric effect of certain LPS compounds on LPS binding to Mono Mac 6 cells.

INDEX WORDS: Endotoxin, Lipopolysaccharide, equine monocytes, *Rhodobacter sphaeroides*, *Rhizobia*, LPS antagonists, transfection, LPS binding
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DEDICATION

To all the teachers who have inspired and supported me in the pursuit of my career.

You continue to be the role models I strive to live up to.

And to my family.
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CHAPTER 1

INTRODUCTION

The studies contained in this report were undertaken to contribute to our knowledge concerning the interactions between endotoxin (lipopolysaccharide, LPS) and inflammatory cells with specific regard to the equine species. Chapter 2 contains a review of the literature and is divided into four sections. Section I describes the nature of endotoxin and briefly discusses the pathophysiology of endotoxemia, specifically taking into account its significance in horses. Section II describes the mechanisms of cellular activation by endotoxin. This section contains a discussion of the concept of pathogen-associated molecular patterns as well as a description of the binding proteins and cellular receptors for endotoxin, the mechanisms of ligand-receptor interactions and the intracellular signaling pathways in response to endotoxin. Section III contains a detailed description of the structure of enteric LPS and LPS from *Rhodobacter sphaeroides* (RsLPS) and Rhizobia, compounds that were evaluated for this report. This section also discusses the literature concerning the relationship between LPS structure and biological activity. Section IV reviews the information concerning LPS antagonists and species-specific differences in the response to LPS compounds. Chapter 3 contains a report investigating the biological activity of RsLPS in equine cells. Chapter 4 expands this investigation by evaluating the basis for differences in response to RsLPS between equine and human cells. Chapter 5 contains an evaluation of two rhizobial LPS compounds with regards to their biological activity in equine and human cells and their interaction with cellular receptor proteins. Chapter 6 finally
describes the evaluation of LPS binding to Mono Mac 6 cells, a human monocytic cell line, as a basis for further investigations.
SECTION I: ENDOTOXEMIA: ETIOLOGY AND PATHOPHYSIOLOGY OF ENDOTOXEMIA

Endotoxin

The discovery of endotoxin is credited to Richard Friedrich Pfeiffer (1858-1945), a German scientist who, while studying *Vibrio cholerae*, described a toxin that was “closely attached to, and probably integral of, the bacterial body.”\(^1\)\(^,\)\(^2\) Pfeiffer observed this toxin to be distinct from the actively secreted, heat-labile and proteinaceous bacterial exotoxins, and endotoxin was later determined to be heat-stable and have a lipopolysaccharide (LPS) structure.\(^3\) The terms endotoxin and LPS are often used interchangeably, although, strictly speaking, the term endotoxin implies immune stimulatory biological activity, while the term LPS represents a mere structural description. Because LPS molecules have been identified that do not activate mammalian immune cells, the term endotoxin should be reserved for agonistic molecules of LPS structure.\(^4\) Throughout this discussion, both terms will be utilized; however, the term endotoxin will only be used in reference to LPS molecules that stimulate inflammatory responses. The outer cell wall of Gram-negative bacteria consists of the inner or cytoplasmic membrane, the periplasm, the outer membrane and the polysaccharide surface layer. With very few exceptions, including *Treponema pallidum* and *Borrelia burgdorferi*, the outer membrane of all Gram-negative bacteria contains LPS as an integral component.\(^5\) LPS is anchored in the outer
membrane via its lipophilic lipid A portion, and represents the major component of the outer
membrane, while the inner membrane consists of phospholipids with inserted bacterial
lipoproteins. The outer cell wall therefore has an asymmetric structure.⁴ LPS in the bacterial cell
wall serves as a permeability barrier against external noxious agents, and the number of LPS
molecules per bacterial cell has been estimated at 3 - 4 x 10⁶.⁵ LPS molecules are released from
the cell wall during bacterial growth and in large numbers upon bacterial death.⁶

LPS molecules typically consist of three portions or regions: the O-chain, the core and
the lipid A portion (Figure 2.1). These regions are genetically and structurally distinct,⁷ and are
conserved among bacterial species to varying degrees. O-chain and core represent the
carbohydrate portion of LPS, while lipid A represents the lipid portion.

Figure 2.1: Structural components of lipopolysaccharide
The O-chain consists of up to 50 repeating oligosaccharide units, each of which is comprised of 2-8 monosaccharide moieties. Among enteric Gram-negative bacteria, the O-chain is highly variable and is responsible for the serologic characteristics of bacterial strains. Colonies of bacteria producing LPS that contains the O-chain have a characteristic “smooth” appearance, which led to use of the term "smooth (S) LPS” for these molecules. In comparison, mutant bacteria with a defect in or lacking the gene cluster responsible for O-chain synthesis exhibit different colony morphology and their LPS is denoted as “rough”.

Rough (R) mutants are further subclassified into Ra, Rb, Rc, Rd and Re based on the length of the remaining core, with Re having the shortest core that is limited to two sugar residues. Although rough LPS mutants are viable in vitro, the O-chain is required for survival of bacteria in host organisms, and enables bacteria to evade phagocytosis and complement-mediated destruction. Bacteria that produce low molecular weight LPS, or lipooligosaccharides, without an O-chain, can be found colonizing mucosal surfaces.

The core region is moderately conserved among species of enteric Gram-negative bacteria and is divided into an outer core and an inner core, which differ in their monosaccharide composition. The outer core mainly contains hexoses including D-glucose, D-galactose, D-glucosamine, N-acetylglucosamine and N-acetylgalactosamine. The inner core consists primarily of L- or D-glycero-D-manno-heptose (L, D-hep) and 2-keto-3-deoxyoctulosonic acid (Kdo ) and represents the most conserved region within the carbohydrate portion of LPS. The lipid A portion is highly conserved; its structure is discussed in more detail in later sections of this chapter. Lipid A is linked to the core region via an acid-labile ketosidic [α-Kdo-(2→6)-D-GlcN(II)] bond between the core proximal Kdo and the 6’ position of lipid A. Mild acid hydrolysis of this bond allows purification of lipid A from LPS preparations. With the O-
polysaccharide and core region supplying hydrophilic properties and lipid A being hydrophobic, LPS is a molecule of overall amphipathic structure, which tends to form aggregates in solution above a critical concentration (critical aggregate concentration or CAC). The CAC is dependent on the degree of hydrophobicity and is thought to be influenced by the number of sugar residues linked to the lipid A portion, as well as the number and distribution of charges. At concentrations exceeding the CAC, equilibrium between free monomers and aggregates of LPS molecules is established, with the CAC determining the number of free LPS molecules. A higher CAC results in more free monomers being present at equilibrium. The CAC is expected to be highest for S-form LPS and lowest for lipid A, which agrees with data showing reduced endotoxic activity of lipid A preparations as compared to LPS.

**Pathophysiology of endotoxemia**

In its literal sense, the term endotoxemia describes the presence of endotoxin in the bloodstream, however it is more customary to associate endotoxemia with the acute systemic pathophysiologic responses to endotoxin. The response to endotoxin is an important component of the mammalian innate defense against Gram-negative bacteria, and successful inflammatory response removes offending pathogens in a locally contained and self-limiting reaction. Clinically relevant endotoxemia on the other hand develops as the result of an unchecked systemic activation of inflammatory cells, which may be attributable to overwhelming amounts of endotoxin or bacteria, or an inability of local inflammatory events to eliminate the offending pathogens. Sensitivity to endotoxin is a characteristic of all mammals but varies distinctly between species. For example, humans, rabbits and ungulates are very susceptible to the effects of endotoxin while mice, rats and hamsters respond much less strongly. In addition to the
species-specific sensitivity, individual response to endotoxin is quite variable. In one study, the minimal stimulatory concentration of LPS in *in vitro* stimulation assays of human mononuclear cells varied among donors by 2 to 3 orders of magnitude.⁹

Sources of endotoxin include Gram-negative pathogens causing infection or Gram-negative bacteria that are part of the endogenous intestinal microflora. Even in health, the host is constantly exposed to small amounts of endotoxin that translocate across the intestinal wall.¹² In healthy humans, plasma endotoxin concentrations of 3-10 pg/ml have been measured, while concentrations exceeded 300 pg/ml in human patients diagnosed with septic shock.¹³⁻¹⁵ Translocated endotoxin is normally taken up and cleared by the mononuclear phagocytic system in the liver, such that it does not activate a systemic inflammatory response. Increased translocation of endotoxin, which overwhelms hepatic removal mechanisms and causes clinical disease, is thought to be responsible for the development of endotoxemia in primarily non-infectious conditions such as severe thermal injury,¹⁶ or any other disease that compromises mucosal barrier function. Translocation mechanisms may be of particular importance in horses, where Gram-negative, anaerobic bacteria represent the major constituent of the endogenous intestinal flora,¹⁷ and acute gastrointestinal disease (or colic) is a common occurrence. Colic, especially if it involves severe mucosal inflammation or intestinal strangulation with a loss of blood supply to the mucosa, therefore ranks highest among the diseases causing endotoxemia in horses. Other conditions commonly associated with the development of endotoxemia in horses are neonatal septicemia and severe septic processes such as pleuropneumonia, pleuritis, peritonitis and endometritis. Measurable plasma concentrations of endotoxin have been detected in 50% of foals with presumed sepsis¹⁸ and in 10 to 40% of colic patients at the time of
Aside from its potential lethality, equine endotoxemia also carries a high risk of permanent damage, most prominently lameness due to laminitis.²¹

Major pathophysiologic events during the development of endotoxemia include the acute phase response; neutrophil activation, margination and extravasation; activation of the coagulation, fibrinolytic and complement cascades; as well as endothelial injury and microvascular failure.²² If allowed to proceed in an uncontrolled fashion, these responses culminate in cardiovascular insufficiency (shock), organ failure and ultimately death. Pathophysiologic events are in part mediated by endotoxin itself, but predominantly by a multitude of cytokines and other inflammatory mediators. These include tumor necrosis factor α (TNFα), the interleukins IL-1, IL-6, and IL-8, platelet activating factor, the arachidonic acid metabolites (prostanoids), kinins, complement components, reactive oxygen species, histamine, and colony stimulating factors which induce leukocyte production.²²-²⁴ Mediators that primarily have an anti-inflammatory effect include IL-10, IL-4 and IL-1 receptor antagonist.²⁵ It is important to recognize that endotoxin stimulates the release of pro-inflammatory as well as anti-inflammatory mediators simultaneously, and that it is the balance of these mediators— or the lack thereof— which determines the outcome of an inflammatory reaction. While endotoxemia is typically interpreted as an excessive stimulation of pro-inflammatory responses, a “compensatory anti-inflammatory response syndrome” has been described and constitutes an overwhelming production of anti-inflammatory mediators leading to a state of immunological anergy and therefore increased susceptibility to infection.²⁶ A “mixed antagonist response syndrome” in which both pro- and anti-inflammatory responses are excessively activated, is further recognized.²⁶
TNFα has been recognized as one of the central mediators of endotoxemia, and therefore is often measured experimentally as a representative indicator of inflammatory cell activation. TNFα is produced primarily by monocytes and macrophages, but also many other cell types including T- and B-lymphocytes, natural killer cells, mast cells and intestinal epithelial cells. TNFα is a proximal mediator produced early in the course of endotoxemia, and in addition to having direct effects it also stimulates the release of other mediators, thereby potentiating the inflammatory cascade. Administration of recombinant TNFα to animals results in many of the same effects as administration of endotoxin, and blockade of TNFα with specific antibodies greatly inhibits the detrimental effects of experimental endotoxin administration. However, blockade of TNFα in some sepsis models failed to improve survival, and anti-TNFα antibodies have not proven useful in reducing lethality in clinical cases of sepsis.

SECTION II: MECHANISMS OF CELLULAR ACTIVATION BY ENDOTOXIN

Pathogen-associated molecular patterns

Pathogen associated molecular patterns, or PAMPs, are molecules that are integral components of pathogenic microorganisms and serve as recognition targets for the mammalian host. PAMPs can be recognized as part of an intact microorganism, such as lipoproteins, can be released from microorganisms, such as endotoxin, or can be freed during bacterial degradation, such as heat shock proteins and 2′-deoxyribo(cytidine-phosphate-guanosine)-rich (CpG-rich) DNA. In effect, PAMPs allow discrimination between "infectious non-self" and "non-infectious self", and evolutionary studies show that receptors for PAMPs developed very early on and
across different phyla that include mammals, insects and even plants.\textsuperscript{34} Toll like receptors (TLRs) have been identified as the mammalian receptors for PAMPs, and are therefore also called pattern recognition receptors. Toll like receptors derive their name from the Toll receptor in Drosophila, which is required for dorso-ventral patterning as well as the defense against fungal infection.\textsuperscript{35} Ten mammalian TLRs have been identified to date and ligands for most of them have been discovered. Some TLRs, such as TLR4 and TLR2, are expressed on the cellular surface, while others, such as TLR7, -8 and -9 reside in the cytoplasm, and are possibly anchored within endosomes.\textsuperscript{36} Aside from their role in early pathogen recognition, it has been suggested that TLRs are operational in "tailoring" adaptive immune responses based on the specific signal they recognize.\textsuperscript{36}

TLR4 is the receptor for endotoxin, but also recognizes lipoteichoic acid and heat shock proteins.\textsuperscript{37, 38} Cellular activation by endotoxin further requires the presence of at least 3 other proteins, namely lipopolysaccharide binding protein (LBP), cluster differentiation antigen 14 (CD14), and MD-2. TLR2 recognizes ligands from Gram-positive and Gram-negative bacteria, mycobacteria, and yeast including peptidoglycan, lipoteichoic acid, bacterial lipoproteins, phenol soluble modulin, lipoarabinomannan and zymosan.\textsuperscript{39-41} TLR9 recognizes CpG-rich DNA,\textsuperscript{41} TLR5 recognizes flagellin from both Gram-positive and Gram-negative bacteria,\textsuperscript{42} and TLR3 recognizes double-stranded RNA from viruses.\textsuperscript{43} Although TLR7 recognizes small anti-viral compounds, an authentic ligand has not yet been identified for this receptor.\textsuperscript{44} TLR1 appears to have no specific ligand but instead interacts with TLR2 and down-regulates its responsiveness. TLR6 acts in concert with TLR2 in the recognition of ligands such as \textit{Borellia burgdorferi} outer surface protein A lipoprotein, and phenol soluble modulin from \textit{Staphylococcus epidermidis}.\textsuperscript{41}
Because of similarities between TLR 1, -6 and –10, TLR10 is thought to interact with TLR2 as well, however, no specific ligand for this receptor combination has been identified.\textsuperscript{41}

Although specific ligands have been identified for different TLRs, the notion that a specific TLR is solely responsible for the defense against an individual class of microorganisms has recently been challenged.\textsuperscript{45} Stimulation of one type of TLR may affect the function of other TLRs, and this phenomenon has been termed "receptor cross talk". For example, endotoxin-activated neutrophils enhance expression of TLR2 on endothelial cells by an oxygen radical-mediated mechanism, thereby enhancing TLR2-mediated up-regulation of endothelial ICAM-1 (intercellular adhesion molecule-1) expression and neutrophil migration upon subsequent stimulation with Gram-positive bacterial products.\textsuperscript{46} TLR2 knockout mice, as compared to wild-type controls, further exhibit reduced alveolar neutrophil migration in response to a single Gram-negative bacterial challenge,\textsuperscript{46} which lends additional support to the hypothesis of “cross talk” between different types of TLRs.

**Binding proteins for lipopolysaccharide**

Inflammatory cells encounter LPS either as an integral component of intact bacteria, or in the form of free molecules. While cell-associated LPS is a target for opsonization, the toxic entity of LPS, namely the lipid A portion, is only able to interact with and activate inflammatory cells after LPS has been released from the bacterial cell.\textsuperscript{24} Because LPS is amphipathic, i.e. it possesses hydrophilic and lipophilic properties, free LPS molecules in solution form aggregates (micelles), which exist in equilibrium with monomeric LPS molecules. Although some evidence suggests interaction of LPS aggregates with cellular receptors,\textsuperscript{47} it is the interaction between monomeric LPS and its receptor that is predominantly responsible for cellular activation.
LBP is a 60 kDa glycoprotein\textsuperscript{48} whose plasma concentration in healthy human volunteers is approximately 10 $\mu$g/ml.\textsuperscript{49-51} LBP belongs to the class of acute phase proteins and increases in concentration approximately 10-100-fold in response to infection or inflammation.\textsuperscript{48, 49} Synthesis of LBP takes place primarily in the liver,\textsuperscript{52} however, extrahepatic sources of LBP production, such as intestinal epithelial cells, lung, kidney and heart have also been identified.\textsuperscript{53, 54} LBP release after exposure to Gram-negative organisms is stimulated directly by LPS as well as by LPS-induced cytokines such as IL-6.\textsuperscript{55} LBP binds both rough and smooth forms of LPS, as well as lipid A and lipid IVa, a lipid A precursor.\textsuperscript{24, 56} LPS-binding to LBP is temperature-sensitive,\textsuperscript{56} and occurs with high affinity (10\textsuperscript{-9} M).\textsuperscript{56} The stoichiometry of binding is 1:1 at low ratios of LPS to LBP, however, large aggregates of LBP and LPS are formed at higher ratios.\textsuperscript{57}

LBP dramatically enhances the potency of LPS, and the sensitivity to LPS of cells in culture increases as much as 100-1000 fold in the presence of LBP.\textsuperscript{58, 59} The mechanism by which LBP enhances LPS-mediated cellular activation is thought to involve removal of monomeric LPS from LPS aggregates and delivery of these monomers to CD14. LBP accelerates binding of LPS to a soluble form of CD14\textsuperscript{57, 60} and transfers LPS monomers from immobilized aggregates to membrane-bound CD14.\textsuperscript{61} Anti-LBP antibodies markedly reduce the potency of LPS; e.g., anti-LBP antibodies reduced TNF\textsubscript{\alpha} production by rabbit whole blood in response to stimulation with LPS from \textit{Salmonella minnesota}.\textsuperscript{58} Anti-LBP antibodies may inhibit the transfer of LPS from LBP to cellular receptors rather than interfere with the initial binding of LPS to LBP, as evidenced by the finding that anti-LBP antibodies prevent binding of LPS to cells even when the antibodies are added after pre-complexing of LPS with serum.\textsuperscript{47}

The sensitizing effect of LBP is mainly operational at low (“resting”) concentrations of LBP. At high concentrations, such as those identified during the acute phase response, LBP
inhibits cellular activation by LPS and aids in the sequestration and removal of LPS in order to prevent the detrimental effects of uncontrolled inflammation. Mechanisms suggested to be responsible for the protective effect of LBP at high concentrations in vivo include increased LPS transfer to high-density lipoproteins (HDL)\textsuperscript{62-64} as well as opsonization and enhanced CD14-dependent phagocytosis of LPS aggregate structures, LPS-bearing particles and intact Gram-negative bacteria.\textsuperscript{65-67} Because of the different effects observed at low and at high concentrations of LBP, the overall biological effect of this protein has been described as bipolar.\textsuperscript{68}

LPS binds to many other proteins, which, like LBP, may play a role in enhancing the cellular activation by LPS, and/or in removing LPS by sequestration and phagocytosis. Bactericidal-permeability inducing-protein (BPI),\textsuperscript{69} a protein of similar structure to LBP, has shown promise as a potential treatment for endotoxemia and sepsis. BPI, which is present in neutrophil azurophilic granules as well as in monocytes and macrophages, is bactericidal for Gram-negative bacteria by increasing outer cell wall permeability and activating enzymes that degrade the bacterial cell wall. These effects of BPI lead to growth arrest, impaired energy metabolism and bacterial cell death.\textsuperscript{70} Binding of LPS by BPI blocks the delivery of LPS to its cellular receptors, inhibits cytokine release by mononuclear cells and inhibits neutrophil activation.\textsuperscript{71-73} BPI competes with LBP for LPS-binding and can prevent excessive activation of the inflammatory response and death from endotoxic shock in animals.\textsuperscript{74} Although plasma concentrations of LBP greatly exceed those of BPI under normal conditions, BPI may play an important role during local infection, as BPI concentrations exceed those of LBP in abscess fluids of human patients.\textsuperscript{51} Clinical studies using the N-terminal domain of recombinant human BPI in septic patients have been promising.\textsuperscript{75}
Other proteins that bind LPS and/or bacteria include lactoferrin, lysozyme, surfactants A and D, serum amyloid P, albumin, transferrin, and hemoglobin. Some of these proteins, such as serum amyloid P, may promote chemotaxis and opsonization, thereby enhancing cellular interaction with LPS. Other proteins may exhibit primarily anti-inflammatory effects by binding and sequestering LPS.

**Cellular receptors**

Presently, three proteins have been identified as components of the major LPS-receptor complex (Figure 2.2).

**Figure 2.2:** Receptors for lipopolysaccharide
CD14 was the first protein to be identified as essential for LPS binding and cellular responsiveness to LPS. CD14 is a 60 kDa, 356 amino acid protein, which contains multiple leucine-rich repeats. The relevance of CD14 for the cellular response to LPS is demonstrated by findings that CD14-deficient mice are resistant to LPS challenge, that transfection of LPS-unresponsive cells with CD14 renders them LPS responsive, that transgenic mice expressing human CD14 are sensitized to LPS, that antibodies directed against CD14 inhibit cellular responses to LPS, and that anti-CD14 antibodies protect animals against experimental challenge with LPS. Binding affinities of LPS-LBP complexes to CD14 have been determined in transfected Chinese hamster ovary (CHO) cells and THP-1 cells, where $K_d$ values ranged from $2.7 \times 10^{-8}$ M to $4.8 \times 10^{-8}$ M. CD14 is constitutively expressed on myeloid cells including monocytes, macrophages, and neutrophils; B lymphocytes; liver parenchymal cells; gingival fibroblasts and microglial cells. CD14 expression differs in cells from different tissues, increases with cell maturation, and can be upregulated rapidly by mobilization of receptors from intracellular stores. It has been estimated that human monocytes constitutively express approximately 115,000 CD14 molecules/cell, whereas human neutrophils express approximately 3,000 CD14 molecules/cell. In addition to membrane-bound CD14 present on the surface of cells, a soluble form of CD14 (sCD14) exists (Figure 2.3). Soluble CD14 can form complexes with LPS that bind to non-CD14-bearing cells such as endothelial and epithelial cells, rendering these cells responsive to LPS. Despite the functional similarity between sCD14 and mCD14, however, the structural determinants of protein function appear to differ. Complex formation between LPS and sCD14 is not dependent on the presence of LBP, however, LBP enhances the formation of LPS-sCD14 complexes. Soluble CD14 is released from monocytes, and the normal concentration of sCD14 in human serum is about 2-6 µg/ml. Serum concentration of
sCD14 increases in patients with septic shock, and is correlated with an increased mortality.\textsuperscript{88} High concentrations of exogenous sCD14 reduce cellular stimulation by LPS \textit{in vitro}\textsuperscript{89,90} and are protective against LPS challenge in mice.\textsuperscript{91} As pathophysiological responses to LPS occur in the presence of high concentrations of endogenous sCD14, however, the potential therapeutic value of sCD14 has been questioned.\textsuperscript{86}

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\caption{Binding of lipopolysaccharide to cells.}
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Despite the indisputable significance of CD14 for LPS binding, CD14 is not the signaling component of the LPS receptor complex. CD14 is a glycosyl-phosphatidyl-inositol (GPI)-anchored protein that lacks a transmembrane domain, and therefore cannot transmit a signal to
the cytosol. Interestingly, however, GPI-anchored LBP that was able to bind LPS to the cellular surface could not substitute for the role of CD14.92 CD14 therefore plays an active role in LPS-induced cellular activation. TLR4 in conjunction with its associated protein MD-2 represents the signal transmitting portion of the LPS receptor complex.93-96 TLR4 was also determined to be the protein encoded by the Lps gene locus responsible for controlling LPS sensitivity.97 TLR4 is a 92 kDa, 841 amino acid type I transmembrane protein that contains leucine-rich repeats in its extracellular portion and has an interleukin 1-receptor-like intracellular domain (Toll/IL-1 receptor domain).98,99 Whereas TIR domains are present in different proteins of many species, and appear to have developed early during the evolution of host defense systems,99 inter- and intraspecies variability in the sequence of TLR4 (and other TLRs) is concentrated on the extracellular portion.4 LPS binding to TLR4 appears to be dependent on the presence of MD-2.100 A point mutation (C→A) leading to an amino acid change (pro→his) within the TIR domain of TLR4 is the reason for LPS hyporesponsiveness in C3H/HeJ mice.101,102 Another LPS-hyporesponsive mouse strain, C57Bl/10ScCr, has a deletion mutation spanning the entire Lps gene locus.102,103 TLR4 is expressed constitutively on neutrophils, monocytes, macrophages and dendritic cells, but also on epithelial and endothelial cells.24 Surface expression of TLR4 on unstimulated human monocytes is relatively low, with approximately 1,300 TLR4 molecules/cell, and there is considerable variation between individuals.104 Exposure to LPS and cytokines changes expression levels of TLR4 differently depending on the tissue. For instance, LPS increases expression of TLR4 in the heart and lungs, while LPS, TNFα and IL-6 decrease TLR4 expression on the surface of macrophages and monocytes.24 Expression of TLR4 also appears to change as cells undergo maturation.104 Although some TLRs appear to interact with their ligands in the cytoplasm, and ligand internalization has been suggested to be essential for
signal transduction in response to LPS, evidence suggests that TLR4-mediated signaling begins by receptor-ligand interaction on the cellular surface. Using fluorescence-protein chimeras of TLR4, it was shown that TLR4 is located both on the cellular surface and in the Golgi, that TLR4 recycles back and forth from the cell surface to the Golgi, that TLR4 trafficking to the Golgi occurs in a complex with CD14 and MD-2, and that TLR4 trafficking to the Golgi is not required for cellular activation. Oligomerization is thought to be required for the function of all TLRs, and MD-2 dependent formation of larger TLR4 aggregates on the cellular surface in response to LPS stimulation has recently been demonstrated. These authors also suggested that TLR4 exists in form of a dimer in unstimulated cells, and that a threshold of aggregated TLR4 molecules must be reached in order to achieve formation of a functional “signalosome”. TLR4-mediated cellular responses may be modified by co-expression of TLR1, which is consistent with findings of a regulatory action of TLR1 and TLR6, respectively, during the activation of TLR2. A soluble form of TLR4 that inhibits LPS-induced signaling has been identified in murine macrophages. This protein is encoded by a splice variant mRNA and may play a role in feedback inhibition of LPS-induced cellular responses.

MD-2 is a secreted 160 amino acid glycoprotein, which associates with TLR4 on the cellular surface. MD-2 binds to both TLR4 and LPS, and LPS binding by MD-2 is enhanced by LBP and CD14. MD-2 may be important for proper glycosylation and transport of TLR4 to the cell surface, as different glycosylation forms of TLR4 occur in the presence and absence of MD-2, however, surface expression of TLR4 in transfected cells has been demonstrated in the absence of MD-2. While one group reported that MD-2 is required for LPS responsiveness of transfected cells, others suggested that TLR4 may be able to effect certain responses, such as
IL-8 production, alone, while requiring MD-2 for other effects such as the activation of mitogen activated protein (MAP) kinases. Mutation of MD-2 induces resistance to LPS stimulation, and one specific mutation (MD-2C95Y) was shown to abolish both the ability of MD-2 to bind TLR4 on the cell surface and to interact with LPS. A requirement for MD-2 in LPS-induced signaling via TLR4 is now generally accepted, although some authors suggest use of knock-out animals to definitively prove an essential role of MD-2.

**Receptor-ligand interactions**

It is generally accepted that cellular activation by LPS results from direct interaction of LPS with its receptor complex. Although this may seem intuitive, it represents a difference from the events in Drosophila, where direct binding of the relevant stimulus to Toll does not occur. Instead, an extracellular serine protease cascade is activated, which serves to produce the actual ligand for Toll. While involvement of extracellular serine proteases in mammalian species similar to the events in Drosophila has been suggested, enough evidence has been put forward to support direct binding of LPS to its receptor proteins. Much of the research concerning LPS binding has focused on the role of CD14, however, recent studies suggest direct interaction between LPS and all of the proteins known to compose the receptor complex.

As discussed earlier in this chapter, LPS is transferred to cellular receptors by LBP, such that binding of LPS to cells can be inhibited both at the level of LBP-LPS complex formation or at the receptor level. For *in vitro* binding experiments that evaluate specific, displaceable binding, this implies that the main target for competition for LPS binding depends on the serum (or LBP) concentration in the assay. At limiting concentrations of LBP, competition for cellular binding is attributable to competition for LBP, while in the presence of excess LBP,
competition for cellular receptor proteins is measured. With respect to the catalysis by LBP of LPS-sCD14 complex formation, both a ternary complex model (all 3 molecules interact)\(^{124}\) and a binary model (LBP transfers LPS monomers to sCD14 but does not itself remain in the complex) have been proposed.\(^{57}\) Following the binding to CD14, LPS is thought to be transferred to TLR4 and MD-2, rather than all three proteins forming a complex that induces cellular activation.\(^{4, 116, 125}\) Thus, LBP and CD14 appear to be primarily responsible for the capture and binding of LPS monomers to the cell surface, while TLR4 and MD-2 represent the signal transducing complex. It has been demonstrated that binding of LPS to the TLR4/MD-2 complex depends on the presence of CD14, and further, that binding of LPS to TLR4 depends on the presence of MD-2.\(^{95, 122}\)

Discrete regions of the plasma membrane in monocytes/macrophages appear to be organized to facilitate LPS binding to CD14,\(^{126}\) and have been identified as lipid rafts. While CD14 is constitutively found within lipid rafts, other proteins that bind LPS, including TLR4, concentrate in lipid rafts following LPS-induced stimulation of cells.\(^{127}\) Signaling molecules such as MyD88 (myeloid differentiation primary response factor 88) have also been detected associated with lipid rafts. Raft-disrupting drugs displace the signaling complex and prevent TNF\(\alpha\) production in response to LPS. Therefore, LPS binding to CD14 appears to serve the function of focusing LPS within lipid rafts in order to allow interaction with several binding proteins and these rafts or microdomains are areas of concentrated receptor signaling.\(^{127}\)

Binding of LPS to cellular receptors is required for the initiation of signaling events; however, binding as such is not sufficient to induce cell signaling. Binding of LPS and cellular signaling can be inhibited with antibodies against CD14,\(^{47, 76, 128-130}\) however, some antibodies
prevent cellular activation without abrogating LPS-binding.\textsuperscript{66} Similarly, cellular signaling events can be inhibited without preventing LPS-binding to TLR4/MD-2.\textsuperscript{122}

### Additional receptors

Several other receptors have been implicated in the binding of LPS and/or cellular responses to an LPS challenge. One family represents the $\beta_2$-integrins, or CD18 antigens, which comprise glycoproteins CD11a/CD18 ($\alpha_1\beta_2$-integrin; leukocyte function-associated antigen 1, LFA-1), CD11b/CD18 ($\alpha_2\beta_2$-integrin; complement receptor type 3, CR3) and CD11c/CD18 ($\alpha_3\beta_2$-integrin, CR4). Several lines of evidence suggest binding of LPS and lipid A as well as intact bacteria by CD18.\textsuperscript{24} Cellular activation by LPS may in part depend on ligand interaction with $\beta_2$-integrins, however, antibodies against these antigens do not entirely inhibit LPS-mediated activation,\textsuperscript{131} and the \textit{in vivo} relevance of the activation pathways may be limited.\textsuperscript{24} In radioligand binding assays, monoclonal antibodies against the CD11/CD18 complex did not inhibit LPS binding to cells and, vice versa, LPS did not inhibit antibody binding.\textsuperscript{128} P-Selectin and L-Selectin are also able to bind LPS.\textsuperscript{132} L-Selectin may be responsible for serum-independent cellular responses to high concentrations of LPS.\textsuperscript{133} A 78 kDa protein that binds LPS and mediates cellular activation has been identified as moesin (membrane-organizing extension spike protein).\textsuperscript{134} Moesin may be involved in the signaling pathway via CD14 and TLR4,\textsuperscript{24} as antibodies against the protein completely inhibit LPS-induced release of TNF$\alpha$ by monocytes.\textsuperscript{134} Class A scavenger receptors (SR-A) are thought to be involved in uptake and detoxification of LPS and lipid A, and in cellular activation of hepatic Kupffer cells.\textsuperscript{135} Aside from SR-A, other scavenger receptors have been identified on Kupffer cells and hepatic
sinusoidal cells, but the relative importance of different scavenger receptors for hepatic LPS uptake has not been resolved.\textsuperscript{24}

In addition to membrane proteins serving as receptors for LPS, cytoplasmatic proteins have been identified that can bind LPS and lead to cellular activation. NOD1 and NOD2 are members of the NOD (nucleotide-binding oligomerization domain) family, whose members are implicated in innate immune responses to bacteria.\textsuperscript{136} Similar to CD14 and TLR4, NOD1 and NOD2 contain leucine-rich repeats in their ligand-recognition domain.\textsuperscript{136} Activation of the transcription factor NF-κB (nuclear factor κB) as well as pro-apoptotic signaling pathways in response to LPS have been demonstrated to involve NOD1 and NOD2.\textsuperscript{137-140} These proteins may therefore confer upon cells the ability to respond to intracellular infection, and may link innate and adaptive immune responses similar to TLRs.\textsuperscript{136}

**Intracellular signaling pathways**

Upon binding of LPS to the TLR4/MD-2 complex, conformational changes in the receptor structure allow recruitment of adapter proteins and a number of kinases that form a signaling complex.\textsuperscript{36} Ultimately, transcription factors such as NF-κB \textsuperscript{93,98} and AP-1 (activator protein 1)\textsuperscript{141} as well as MAPK\textsuperscript{142} are activated, resulting in increased transcription of pro- and anti-inflammatory genes (Figure 2.4). Signaling pathways in response to binding of LPS to TLR4/MD-2 are very similar to those initiated in response to activation of IL-1 receptors. Because TIR domains are present in all TLRs, similarities further exist between signaling events activated by different receptors.

MyD88,\textsuperscript{143} Mal (MyD88 adapter like; also called TIRAP, Toll/IL-1 receptor domain containing adapter protein)\textsuperscript{144} TRIF (TIR domain containing adapter-inducing interferon (IFN)-
β; also called TICAM-1, TIR-containing adapter molecule-1)\textsuperscript{145,146} and TICAM-2\textsuperscript{147} have been identified as initial adapter proteins involved in signaling via TLR4. MyD88-dependent pathways are responsible for signaling events that result in increased cytokine production, while an MyD88-independent pathway is involved in activation of IRF-3 (IFN-regulatory factor-3) and induction of IFN-β and IFN-inducible genes.\textsuperscript{148} Mal was initially thought to initiate the MyD88-independent pathway,\textsuperscript{149} however, it was later shown to be operative in MyD88-dependent

**Figure 2.4:** Signaling pathways in response to LPS-receptor activation.

signaling pathways via both TLR2 and TLR4.\textsuperscript{150} MyD88-independent signaling is now thought to be attributable to TRIF\textsuperscript{146} and its bridging adapter TICAM-2.\textsuperscript{147} The adapter proteins bind to
the intracellular portion of TLR4 via homophilic interaction of TIR domains located on both the receptor and the adapter protein. Aside from signaling via TLR4, MyD88 is implicated in signaling via multiple other receptors, including TLR2 and other TLRs, and TRIF may play a role in signaling via TLR3.99, 144, 146, 151

After MyD88/Mal binds to TLR4, several kinases and additional adapter proteins are recruited to form a signaling cascade. IRAK-1 (IL-1 receptor associated kinase), IRAK-4 and IRAK-2 are proximal kinases that are recruited to MyD88 and are subsequently phosphorylated. IRAK-2 binds only weakly to TLR4 as compared to IL-R1, suggesting a minor role for IRAK-2 in TLR4-mediated cell signaling.153 In unstimulated cells, IRAK is bound to Tollip (Toll-interacting protein), and dissociation from this protein is necessary to allow phosphorylation and activation of IRAK.154 Over-expression of Tollip inhibits IRAK phosphorylation and kinase activity as well as NF-κB activation in response to activation of IL-1β-, TLR2- and TLR4-receptors.155, 156 The physiological significance of Tollip has not been unequivocally proven, however, it may serve to maintain immune cells in a quiescent state in the absence of infection, and to allow termination of signaling in the event of cellular stimulation during inflammatory events.156 After IRAK phosphorylation, the adapter protein TRAF-6 (TNFα receptor associated factor-6)157, 158 is recruited and activated, and via multiple kinase steps leads to activation of the IKK (I-κB kinase) complex. TRAF-6 acts in combination with two activator complexes; TRIKA1 (TRAF-6-regulated IKK activator 1), consisting of the ubiquitinating enzymes Ubc13 and Uev1A, and TRIKA2, which encompasses TAK-1 (transforming growth factor (TGF)β-activating kinase, a MAP kinase)159 with its associated proteins TAB1 (TAK –1 binding protein 1) and TAB2.160, 161 Activated IKK phosphorylates I-κB (inhibitory factor κB), which results in the proteasome-mediated degradation of I-κB. Because I-κB sequesters NF-κB in unstimulated
cells, its degradation frees NF-κB and enables it to translocate into the nucleus and act as an enhancer of gene transcription.\textsuperscript{162}

MAP kinase activation pathways utilize MyD88 and TRAF-6,\textsuperscript{158} and further involve Ras/Raf-1/MAP kinase kinase (MEK) for ERK1 (extracellular regulated kinase 1) and ERK2 activation, MEKK-1/MEK-4 for JNK (Jun N-terminal kinase) activation, and MEK-3 for p38 activation.\textsuperscript{163, 164} TAK-1 is involved in MAP kinase activation by activating kinases upstream of p38 and JNK.\textsuperscript{161} MAP kinase activation leads to activation of transcription factors such as AP-1, ATF-2 (activating transcription factor 2), c-Jun, c-fos and Elk1, and results in stabilization of mRNA containing AU repeats.\textsuperscript{141, 165, 166} In RAW 264.7 cells, a murine macrophage cell line, activation of MAPK pathways by itself induces TNFα production and secretion, however, additional signals, such as activation of NF-κB are necessary to fully stimulate TNFα production.\textsuperscript{167, 168}

Although many steps of the signal transduction pathways in response to LPS have been elucidated, some LPS-responsive elements have not yet been definitively attributed to certain steps in the pathways. For example, atypical protein kinase (PKCζ) interacts with TRAF-6, and macrophages from PKCζ-deficient mice exhibit reduced cytokine production in response to endotoxin stimulation.\textsuperscript{169, 170} A new gene locus, namely Lps2 (in distinction of Lps encoding for TLR4) has also been described, which upon mutation significantly decreases cellular response to LPS, although its effect is not as severe as that of Lps mutation.\textsuperscript{171, 172} The description of signal transduction events in response to LPS is therefore a work in progress, and additional signaling proteins will likely be discovered. In addition, it is possible that signaling events differ between cell types.\textsuperscript{173}
Aside from activating intracellular signaling pathways and causing inflammatory cell activation, LPS binding to cells can result in internalization of the ligand. Both cellular activation and ligand internalization are mediated by binding of LPS to membrane CD14 but represent separate events that can be inhibited individually using different monoclonal antibodies. Internalization is conserved in LPS-hyporesponsive cells and after inhibition of signaling events by LPS antagonists. Mechanisms of LPS internalization include micropinocytosis (non-coated plasma membrane invaginations), macropinocytosis (phagocytosis), formation of clathrin-coated pits (receptor-mediated uptake) and plasma membrane penetration. The mode and rate of internalization appears to differ based on LPS structure, and aggregation of LPS promotes accelerated entry of LPS into monocytes via a non-coated pathway.

SECTION III: STRUCTURE OF LIPOPOLYSACCHARIDE AND STRUCTURE-ACTIVITY RELATIONSHIPS

As mentioned earlier in this chapter, the terms endotoxin and LPS are frequently used interchangeably. Once LPS molecules other than those derived from pathogenic, enteric bacteria are discussed, however, this terminology can lead to confusion because, while they all have LPS structure, biological effects of LPS molecules vary depending on the bacterial species and also depending on the host species. In the following section, therefore, LPS molecules that effect activation of inflammatory cells will be referred to as endotoxin agonists or simply agonists, or as having endotoxic activity. LPS molecules that bind to LPS receptors without evoking cellular activation, and that block the cellular response to agonist LPS, will be referred to as endotoxin antagonists or LPS antagonists. It might be more appropriate to refer to these compounds as LPS
receptor agonists or antagonists, however, this terminology would suggest that all compounds of LPS structure exert their effects via the same receptor, which has not been unequivocally proven.

Lipopolysaccharide from enteric bacteria

Lipid A has been identified as the toxic principle of LPS\textsuperscript{177, 178} and its toxicity is dependent on certain structural features. Lipid A from \textit{Escherichia coli} (\textit{E. coli}) is considered to be representative of enteric lipid A and exhibits the highest toxicity. This lipid A consists of a diglucosamine backbone in $\beta(1 \rightarrow 6)$ linkage, which is phosphorylated at the 1- and 4’-position and is substituted asymmetrically with 6 fatty acyl chains (hexaacylated LPS) (Figure 2.5). Positions 2, 3, 2’ and 3’ are substituted with primary (R)-3-hydroxytetradecanoyl (myristoyl, 3-OH-C14:0) groups, with the acyl chains being amide-linked in positions 2 and 2’ and ester-linked in positions 3 and 3’. Two of the primary myristoyl groups carry ester-linked secondary acyl chains, a myristoyl (C14:0) group in position 3’ and a lauroyl (dodecanoyl, C12:0) group in position 2’, and are therefore denoted as acyloxyacyl chains.\textsuperscript{7}

With regards to the 3-dimensional structure of lipid A, evaluation of the crystal structure of \textit{E. coli} lipid A shows that the proximal polar portion (backbone and proximal acyl chains) is approximately 6 Å in length, while the hydrophobic portion of the acyl chains contributes about 12-14 Å in length.\textsuperscript{179, 180} Two backbone conformations appear to be present; in one, the glucosamine residues are oriented orthogonally, in the other, the backbone takes on a twisted conformation, which leads to a diametrically opposed arrangement of the secondary acyl chains. Because of potential solvent effects during analysis, however, lipid A conformation may be different in aqueous solutions.\textsuperscript{7}
Figure 2.5: Structure of lipid A from *E. coli*.

The basic structural features of lipid A, i.e. the backbone with charged substitutions and the acylation with primary and secondary fatty acyl groups are present in all Gram-negative bacteria. However, variations from the “typical” enteric lipid A as described above are common. Variations occur non-stoichiometrically within serotypes of bacteria, such that natural LPS preparations represent inherently heterogeneous mixtures, and between bacterial species. Heterogeneity of lipid A preparations from an individual bacterial strain is attributable to incomplete biosynthesis, or may represent a preparation artifact due to partial degradation processes. In enteric bacteria, certain growth conditions further elicit LPS modifications, which are presumed to increase the virulence of an organism. Structural variations may affect the lipid A backbone and/or the acyl chain substitutions. Backbone alterations include replacement...
of one or both of the backbone monosaccharides with D-2,3-diamino-2,3-dideoxy-glucose (D-GlcN3N; 2,3-diaminoglicosamine, DAG), as well as alternative or extended substitutions in the 1- and 4'-position such as pyrophosphate, phosphoethanolamine, diphosphoethanolamine, phosphate-esterified L-arabinosamine or L,D-glycero-D-manno-heptose, and D-galacturonic acid.\(^4\) Backbone substitution beyond phosphorylation can occur at varying percentages in natural lipid A.\(^7\) Variation in the acylation pattern can present as hydroxylation of acyl residues, or may affect the length and type of acyl chains, the symmetry of acyl substitution or the number of acyl residues. The primary acyl chains are more conserved between species than the secondary chains, and almost always represent (R)-3-hydroxy or 3-oxo fatty acids.\(^7\) Secondary acyl chains may be unsaturated fatty acids, long chain fatty acids, 2-hydroxylated, (n-1)-hydroxylated and (n-1)-oxo fatty acids, and may contain additional unsaturated bonds.\(^7\) Lipid A from \textit{Haemophilus influenzae} and \textit{Klebsiella pneumoniae}, e.g., carry two secondary myristoyl groups\(^{182,183}\) instead of one myristoyl and one lauroyl group in \textit{E. coli} lipid A. Despite these variations, however, chemical analysis of lipid A from different species shows that at least one acyloxyacyl group is always present.\(^{184}\) In LPS preparations obtained from \textit{Salmonella enterica} sv. Minnesota, a fraction of molecules is heptaacylated and carries an acyloxyacyl chain in position 2.\(^{185}\) This structure is referred to as \textit{“Salmonella-type”} LPS in distinction from the hexaacylated \textit{“E. coli-type”} LPS.

\textbf{Lipopolysaccharide from \textit{Rhodobacter sphaeroides} and Rhizobia}

\textit{Rhodobacter sphaeroides} is a facultative phototrophic bacterium belonging to the subgroup \textit{Proteobacteria}. The structure of lipid A from \textit{Rhodobacter sphaeroides} has been resolved\(^{186,187}\) and is shown in Figure 2.6. \textit{Rhodobacter sphaeroides} lipid A has the same
backbone structure as *E. coli* lipid A (β-D-glucosaminly-1,6-D-glucosamine) with phosphate groups at the 1- and 4’-positions of the disaccharide. However, *Rhodobacter sphaeroides* lipid A differs with regard to the number, type and location of its fatty acyl chains. It is a pentaacylated lipid A with only one acyloxyacyl chain (Δ7-tetradecenoyltetradecanoate, Δ7-C14OC14), which is N-linked in position 2. The other fatty acyl chains are 3-oxotetradecanoyl (3-O-C14:0) in position 2’ (N-linked), and 2-hydroxydecanoyl (3-OH-C10:0) in position 3 and 3’ (ester linkage).

![Structure of lipid A from *Rhodobacter sphaeroides*.](image)

**Figure 2.6:** Structure of lipid A from *Rhodobacter sphaeroides*.

Rhizobia are Gram-negative bacteria belonging to the family of *Rhizobiaceae*, which are nitrogen fixing bacteria that form symbiotic relationships with legume plants in the form of root nodules. The lipid A structure of LPS produced by these bacteria differs considerably from the
structure of enteric lipid A, and has been elucidated for several species. *Rhizobium etli* and *Rhizobium leguminosarum* have very unusual lipid A structures. The backbone is a trisaccharide consisting of β-glucosamine, 2-aminogluconate and galacturonic acid. The backbone lacks phosphate, however, the substituents at the 1- and 4’-end are negatively charged, which is analogous to the backbone of enteric lipid A. The galacturonic acid residue is not acylated, but all available positions in the other 2 monosaccharides are acylated heterogeneously with 3-OH-C14:0, 3-OH-C16:0, 3-OH-C18:0, 3-OH-C15:0 or 27-OH-C28:0. The only acyloxyacyl chain is 27-OH-C28:0, which carries an ester-linked secondary β-hydroxybutyroyl at the 27-hydroxy position. This long chain 27-hydroxyoctacosanoid acid residue is common in lipid A structures derived from Rhizobia.

Lipid A extracted from *Rhizobium species Sin-1 (R. Sin-1)* yields a mixture of structures differing in the disaccharide backbone and the acylation pattern (Figure 2.7). Two backbone types have been identified: one consists of a distal glucosamine residue linked to the 6-position of a proximal 2-aminoglucono-1,5-lactonosyl residue (shown in Figure 2.7); the other contains a distal glucosamine residue linked to the 6-position of a proximal 2-amino-2,3-dideoxy-D-erythro-hex-2-enono-1,5-lactone residue (2-aminoglucono-2-ene-1,5-lactone). The ratio of these two lipid A species is approximately 2:3. Consistent with the structures of lipid A of *R. etli* and *R. leguminosarum*, the backbone lacks phosphate. With regards to acylation, several patterns exist; the predominant structures are shown in Figure 2.7. In both lipid A species, position 2’ is substituted with β-hydroxypalmitoyl (3-OH-C16:0) that is O-acylated by a secondary 27-hydroxyoctacosanoid acid (27-OH-C28:0). Position 3’ is either acylated with 3-hydroxymyristoyl (3-OH-C14:0) or does not carry an acyl chain. Both types of proximal residue are substituted in position 2, predominantly with 3-OH-C16:0. A small fraction of lipid A
Figure 2.7: Predominant structures of lipid A from *Rhizobium Sin-1*. The arrows point out fatty acyl substitutions that vary among the structures.
molecules is substituted with 3-OH-C14:0 in position 3 and/or 3-hydroxystearoyl (3-OH-C18:0) or 3-hydroxyoctadecenoyl (β-OH-C18:1) in position 2 and 2’.191

**Structure-activity relationships**

Smooth LPS and rough mutants from *Salmonella abortus equi* and *Salmonella minnesota*, respectively, do not differ with regard to their IL-1 inducing potency in human mononuclear cell preparations.9 Synthetic lipid A, on the other hand, consisting solely of the acylated, bis-phosphorylated disaccharide backbone and lacking any core structure, is approximately 10-100 fold less potent than LPS in inducing IL-1 production.9 The Re mutant therefore is the minimal LPS structure needed for full endotoxic activity, while lipid A is recognized as the endotoxic principle of LPS.177, 192 The difference in potency between LPS and lipid A may be attributable to the difference in CAC between these structures, as discussed above. Although core oligo-trisaccharides (Hep-Hep-KDO) by themselves have little to no capacity to stimulate inflammatory responses,9 the LPS core is thought to modulate the biological activity of lipid A.7

Structural determinants of the endotoxic activity of lipid A have been identified by comparing the endotoxic potency of “typical” enteric lipid A with natural variants, chemically modified lipid A or lipid A partial structures. Each structural component (i.e. the intact disaccharide backbone, the substituting groups at the 1- and 4’-ends, and the acylation pattern) appears to be relevant.4 Removal of either phosphate from the backbone of *E. coli* lipid A reduces its IL-1 inducing potency in human mononuclear cell preparations 10-100-fold, with removal of the 4’- phosphate reducing lipid A potency less than removal of the 1-phosphate in most experiments.9 Reduced backbone acylation dramatically decreases the IL-1 inducing
capacity of lipid A in these cell preparations such that pentaacylated lipid A is $10^5$- to $10^6$-fold less active than hexaacylated lipid A. An engineered mutant of *S. enterica* sv. Typhimurium that produces pentaacylated lipid A was nontoxic in a mouse typhoid model. Lipid IVa, a synthetic tetraacyl lipid A precursor without acyloxyacyl chains, bis-acylated disaccharide compounds and lipid X (2,3-diacylglucosamine-1-phosphate), a monosaccharide precursor of lipid A, are inactive. Increased acylation, as in heptaacylated *Salmonella*-type lipid A, also reduces agonist activity as compared to hexaacylated *E. coli*-type lipid A. Although hexaacylation therefore appears to be important for the endotoxic activity of enteric lipid A, non-toxic hexaacylated lipid A also exists, for example in the non-enteric *Chromobacterium violaceum*. The effect of decreased or increased acylation further appears to be species-specific, as heptaacylated *Salmonella*-type lipid A is an agonist in murine peritoneal macrophages and murine 1774-1 cells, and pentaacylated *Rhodobacter sphaeroides* lipid A is an agonist in hamster cells. In addition to the number of acyl chains, the effect of symmetrical or asymmetrical patterns of acylation has been investigated. Again, no general rule can be established and symmetrical backbone substitution has been identified in both toxic (e.g. *Neisseria meningitidis*) and non-toxic (e.g. *Chromobacterium violaceum*) lipid A.

A different approach in the evaluation of structure-activity relationships of LPS has focused on the overall shape of lipid A. The basis for this approach is a model of LPS-induced activation that proposes direct intercalation of LPS (or lipid A) into cellular phospholipid membranes, and their uptake into the cell interior, as essential steps during endotoxin-induced cell signaling. According to this model, membrane intercalation is accompanied by specific recognition of LPS or lipid A by membrane receptors, and signaling is induced by steric stress after lateral diffusion of agonist molecules to transmembrane receptor proteins. Antagonists are
those molecules that are not able to induce the steric stress, but block sites surrounding the sensitive proteins.\textsuperscript{197} According to the proponents of this model, endotoxic activity correlates with the aggregate, or micellar, structure exhibited by a particular LPS or lipid A.\textsuperscript{8} Aggregate structure is determined by the acylation of lipid A, as well as temperature and ionic strength, and therefore varies between bacterial serotypes.\textsuperscript{24} Aggregate structure is evaluated by radiation X-ray diffraction technique, and has been described as being lamellar, cubic or hexagonal.\textsuperscript{8} From the aggregate structure, the shape of individual lipid A molecules is then inferred. Lipid A shape refers to the relationship of cross-sectional diameters of the hydrophilic (backbone and proximal polar regions of acyl chains) as compared to the hydrophobic (distal acyl chains) region. In non-lamellar inverted aggregate structures (called cubic Q or hexagonal H\textsubscript{II} structures), the hydrophobic portion has a larger cross-section than the hydrophilic portion and the lipid A shape is conical. In lamellar structures, both cross-sections are equivalent, and the lipid A molecule has a cylindrical shape (Figure 2.8).\textsuperscript{200, 201}

Only conically shaped lipid A molecules are thought to be biologically active endotoxins that stimulate inflammatory cells, and this shape has been termed the endotoxic conformation.\textsuperscript{8, 197, 202} In support of this terminology, enterobacterial hexaacyl lipid A exhibits a conical/concave shape, while pentaacyl lipid A, tetraacyl lipid A and lipid A derived from non-enteric bacteria are cylindrical and induce minimal to no production of IL-6.\textsuperscript{197} Further, addition of chlorpromazine, which converts inactive lipid As into agonists,\textsuperscript{106} converts the pentaacyl lipid A from \textit{E. coli} from a cylindrical to a conical/concave shape, and changes its biological activity from an antagonist to an agonist.\textsuperscript{197} Conversely, removal of the glycosidic phosphate from ReLPS of \textit{E. coli} changes the aggregate form from cubic to lamellar/cubic, and reduces the endotoxic activity of its lipid A. Additional removal of the second phosphate results in a
Figure 2.8: Lipid A shape. Lipid A molecules can assume conical (e.g. *E. coli* lipid A), cylindrical (e.g. lipid IVa) or intermediary shapes. Only conically shaped lipid A are thought to have endotoxic activity.

Given the species-specific responses to some atypical lipid A structures such as that from *Rhodobacter sphaeroides*, definition of an endotoxic conformation may need to be specified for each species.

Some lipid A molecules, such as those from *Rhodospirillum fulvrum* and *Rhodomicrobium vannielii*, apparently do not bind to cellular LPS receptors at all and therefore
are neither agonists nor antagonists. The lipid A backbone structure, specifically a sufficiently high negative charge density, is thought to determine binding of lipid A to LBP and subsequently to cellular receptors. Atypical backbone structures may affect LPS activity by at least two mechanisms. First, replacement of highly negatively charged phosphate residues with lower-charged residues such as galacturonic acid in *Rhodospirillum fulvrum*, or a complete lack of negative charges, as found in *Rhodomicrobium vannielii*, may prevent proper receptor-ligand interaction. Second, while small phosphate groups allow the molecule to assume a conical shape, large charge-carrying groups such as galacturonic acid result in a cylindrical shape and may in this manner interfere with LBP-mediated transport and reduce endotoxic activity.

**SECTION IV: LIPOPOLYSACCHARIDE ANTAGONISTS AND SPECIES-SPECIFIC DIFFERENCES IN THE RESPONSE TO LIPOPOLYSACCHARIDE COMPOUNDS**

**General concepts**

Endotoxin antagonists are non-toxic molecules of LPS structure that block cellular responses to agonist LPS. Typically, endotoxin antagonists exhibit distinct structural variation from enteric LPS. The mechanism of antagonism is thought to be mostly competitive, thereby implying that LPS antagonists interact with the same targets as agonist LPS. Endotoxin antagonists are of great interest from a clinical as well as research standpoint. They present novel therapeutic options, and have yielded promising results in some clinical studies. In addition, endotoxin antagonists provide a research tool for the investigation of ligand-receptor interactions. LPS antagonists that have been investigated most extensively include lipid X,
deacylated (tetraacylated) LPS and its synthetic analogue lipid IVa, and LPS or lipid A from
*Rhodobacter sphaeroides.*

As has been mentioned previously, some LPS can exert agonistic or antagonistic effects
depending on the host species (Table 2.1). It has been proposed that species-specific responses
are determined by the structure of one or more of the LPS receptor proteins, and individual
proteins have been implicated with respect to certain LPS structures. In order to obtain chimeric
receptor complexes that comprise proteins of different species origin, transfection experiments
are often employed in these investigations. One common method utilizes over-expression of
individual LPS receptor proteins of one species in LPS-responsive cells of a different species,
and subsequent evaluation of the cells’ phenotype, i.e. their recognition of a given LPS as an
agonist or an antagonist. Agonist recognition in these cells can be determined by evaluating
"natural" responses to LPS such as cytokine production in mononuclear cells or adhesion
molecule expression in neutrophils. Antagonist recognition is determined by evaluating the
effect of the LPS ligand on cellular recognition of agonist LPS. Alteration of the cellular
phenotype is attributed to an effect of the protein that is encoded by the transfected gene. A
variation of this approach uses protein expression in cells of mutant animals that lack expression
of a particular protein. For example, the effect of TLR4 can be evaluated by expressing this
protein in cells from C3H/HeJ mice or C57BL/10ScCr mice that lack functional endogenous
TLR4. An alternative transfection process employs protein expression in cells that are inherently
unresponsive to LPS, such as human embryonic kidney (HEK) cells. Successful expression of an
LPS receptor complex confers responsiveness to LPS to these cells, however, no "natural"
cellular responses can be measured. Instead, reporter assay systems are used, which measure
activation of transcription factors such as NF-κB.
Table 2.1: Biological activity of certain LPS/lipid A compounds in different species. A = LPS antagonist, T = toxic (agonistic) LPS. [A]: no stimulation by LPS but antagonistic effect not shown. *R. sphaeroides* = *Rhodobacter sphaeroides*, *B. lupini* = *Bradyrhizobium lupini*, *R. meliloti* = *Rhizobium meliloti*, BM = bone marrow, perit. MØ = peritoneal macrophages, nr = not reported.

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Species-specific effects of different lipopolysaccharide compounds

Lipid X, a diacylated monosaccharide precursor of enteric lipid A is an antagonist in human and murine cells *in vitro*, and protects mice and sheep from the lethal effects of an endotoxin challenge. Initial reports of agonistic effects of lipid X were later attributed to contamination of lipid X preparations with immunostimulatory molecules. Despite the protection against *in vivo* endotoxin challenge, a trial in dogs using an *E. coli* infectious septic shock model showed no benefit of lipid X administration.
Deacylated (tetraacylated) LPS and its synthetic analog, lipid IVa, inhibit endotoxin-induced upregulation of CD11b/CD18 expression on human neutrophils. Lipid IVa further prevents the release of TNFα and prostaglandin (PG)E₂ by human THP-1 cells in response to Salmonella LPS and intact bacteria, prevents endotoxin-induced production of TNFα, IL-1β and IL-6 by isolated human mononuclear cells, and prevents TNFα production in human whole blood *ex vivo*. In human umbilical vein endothelial cells, lipid IVa inhibits endotoxin-induced expression of tissue factor. In contrast to its activity in human cells, lipid IVa is an agonist in murine and hamster cells. In murine RAW 264.7 cells and native murine peritoneal macrophages, lipid IVa stimulates the release of TNFα. Lipid A from *Rhodobacter sphaeroides*, which also prevents cellular activation by enteric LPS, inhibits activation of RAW 264.7 cells and of the murine lymphoma cell line 70Z/3 by lipid IVa, which suggests that all molecules interact with the same cellular targets. Competition by lipid IVa for LPS binding to cells has further been demonstrated in human monocytes, where lipid IVa bound with similar affinity as an unlabeled rough ReLPS.

LPS (RsLPS) and lipid A (diphosphoryl-lipid A, RsDPLA) from *Rhodobacter sphaeroides* act as antagonists in human and murine cells, but stimulate hamster cells similarly to enteric LPS. Initially it was suggested that the lack of toxicity of either LPS or lipid A from *Rhodobacter sphaeroides* in human and murine cells could be attributable to the presence of unsaturation in Δ⁷-C₁₄OC₁₄ and the keto group in 3-O-C₁₄:0. However, these structural features were later shown not to be responsible for the antagonistic activity of RsDPLA. RsDPLA potently antagonizes LPS-induced cytokine production in human and murine cells. Specifically, RsDPLA inhibits release of TNFα and PGE₂ by human THP-1 cells and release of TNFα, IL-1β and IL-6 by freshly isolated human mononuclear cells, inhibits TNFα-
production and blocks binding and internalization of LPS in murine RAW264.7 cells, and inhibits production of TNFα and IL-1β in murine peritoneal macrophages. Most likely, a competitive mechanism is responsible for the inhibitory effects of RsDPLA, because it is overridden by increasing concentrations of LPS or bacteria. Cytokine release in vitro is typically inhibited at a 10- to 100-fold excess of RsDPLA over enteric LPS. Inhibition of cellular responses to intact bacteria has also been demonstrated, and RsDPLA prevents LPS-induced mortality in mice. Other effects attributable to RsDPLA are inhibition of macrophage tolerance to LPS stimulation in vitro, inhibition of pre-B-cell activation, and inhibition of LPS-induced surface expression of CD11b/CD18 on human neutrophils.

RsDPLA inhibits binding of radioiodinated E. coli LPS to a 73-kDa receptors on murine lymphoreticular cells and the murine 70Z/3 cell line, and to the murine macrophage-like cell line J774.1. RsDPLA is at least equally potent as unlabeled rough (Re) enteric LPS in inhibiting LPS binding. RsDPLA competes with LPS for binding to LBP and soluble CD14 at a 1:1 ratio suggesting the same or similar binding sites. Some evidence suggests that RsDPLA is not merely a competitive endotoxin antagonist, but that it exerts some stimulating effects and that mechanisms other than competitive inhibition are responsible for its antagonistic activity. RsDPLA suppresses the induction of several LPS-induced genes (e.g. TNFα, IL-1β) as well as protein-tyrosine phosphorylation in murine peritoneal macrophages, however, high concentrations of RsDPLA (20µg/ml) induce moderate expression of TNFα receptor-1 and TNFα mRNA. RsDPLA also effects transient NF-κB activation in 70Z/3 cells, and has an inhibitory effect on T-cell suppressor activity similar to enteric LPS. In mice, suppression by RsDPLA of endotoxin-induced TNFα production and lethality is dependent on the presence of a functioning adrenal gland, and high doses of RsDPLA induce corticosterone production in
healthy mice. It has therefore been argued that, in addition to competitive inhibition of LPS binding and LPS-induced cell activation, beneficial effects of RsDPLA may result from an activation of anti-inflammatory responses without concurrently activating the pro-inflammatory axis.

Based on the structure of LPS from *Rhodobacter capsulatus*, an LPS of similar structure to RsLPS, the more stable compounds E5531 and E5564 have been designed and are now in clinical trials as LPS antagonists. E5531 blocks LPS binding and inhibits LPS-induced TNFα production in human and murine cells, inhibits the induction of fever by *E. coli* LPS in rabbits, and is a potent LPS antagonist in endotoxin challenge tests in human volunteers. E5531 is thought to directly antagonize endotoxin and not act via the induction of glucocorticoids or other mediators. Similar to E5531, E5564 blocks cytokine production in vitro and in vivo, and prevents the development of clinical signs and symptoms in human volunteers administered endotoxin. E5564 has an extended duration of activity than E5531 and is therefore more likely to become a clinically useful endotoxin antagonist.

Information about the biological activity of LPS or lipid A of rhizobial bacteria is relatively sparse and suggests differences between species of bacteria as well as between mammalian species exposed to the compounds. LPS from *Bradyrhizobium lupini* and from *Rhizobium meliloti* stimulate production of TNFα, IL-1 and IL-6 in human mononuclear cells and are lethal for galactosamine-sensitized mice. For comparison purposes, the lethal dose of *Salmonella abortus-equii* LPS is approximately the same as for these rhizobial compounds, whereas *Rhodobacter capsulatus* LPS is 1000-fold less potent. LPS from *Rhizobium leguminosarum* induces the production of TNFα, IL-1β, IL-6 and IFNγ in murine splenocytes, and in mice is almost as lethal as LPS from *Salmonella typhimurium*. In comparison, LPS from
*Mesorhizobium loti* has a 1000-fold reduced lethality and cytokine-inducing capacity.\textsuperscript{241} With regard to structure-activity relationships for rhizobial compounds, the unusually long fatty acyl chain in *Bradyrhizobium lupini* and *Rhizobium meliloti* purportedly compensates for a lower degree of acylation by increasing the hydrophobicity of lipid A.\textsuperscript{240} Some findings suggest that LPS from rhizobial bacteria may interact with more than one receptor type, possibly dependent on the cell type under investigation. LPS from *Rhizobium Sin-1* does not induce TNF\(\alpha\) production in murine peritoneal macrophages\textsuperscript{242} and inhibits *E. coli* LPS-induced TNF\(\alpha\) production in human monocytic cells.\textsuperscript{243} LPS from *Rhizobium Sin-1* competes with *E. coli* LPS for binding to LBP and to monocytes,\textsuperscript{243} which implies shared recognition sites. In contrast, LPS from *Rhizobium leguminosarum bv. trifolii 24 AR, Rhizobium galegae* and *Rhizobium Sin-1* induce CD14 expression by murine bone marrow granulocytes independent of TLR4,\textsuperscript{244} and the constitutive receptor responsible for the induction of CD14 has been identified as TLR2.\textsuperscript{242} In support of the results of binding studies performed with monocytes, rhizobial LPS competes with enteric LPS for binding to the induced CD14 on bone marrow granulocytes.\textsuperscript{244} The implication that certain rhizobial LPS molecules are able to exert biological effects via TLR2 is consistent with experiments using LPS from bacteria such as *Porphyromonas gingivalis*,\textsuperscript{245} *Leptospira interrogans*,\textsuperscript{246} and *Legionella pneumophila*.\textsuperscript{242} The presence of a substituent or branch of the penultimate carbon of a fatty acyl chain may be the structural characteristic that determines TLR2-mediated effects of these compounds.\textsuperscript{242} Interestingly, LPS from *Bordetella pertussis* inhibits binding of rhizobial LPS and cell activation via TLR2 in bone marrow granulocytes, while its own activity depends on TLR4.\textsuperscript{244} *Bordetella pertussis* LPS does not prevent binding of non-LPS TLR2 ligands such as lipoproteins, suggesting alternative binding sites on TLR2 for structurally diverse ligands.\textsuperscript{244}
Determination of species-specific responses to lipopolysaccharides by individual receptor proteins

Transfection studies have implicated individual receptor proteins in species-specific responses to certain LPS, however, contradictory results have sometimes been obtained and the final conclusions have yet to be drawn. CD14 is very likely not responsible for discrimination between agonist and antagonist ligands, which is not surprising given its important role in ligand binding but not signal transduction. Over-expression of human or murine CD14 in different cell types increases the overall responsiveness of cells to various LPS, but the origin of CD14 does not alter the transfected cells' phenotype. The roles of TLR4 and/or MD-2 in the response to LPS receptor ligands have not been elucidated completely. Several studies suggest that TLR4 determines the cellular phenotype. Macrophages from C3H/HeJ mice (expressing endogenous CD14 and MD-2 but dysfunctional TLR4) that are transfected with murine TLR4 recognize lipid IVa as an agonist, while those transfected with human TLR4 do not. Similarly, CHO cells that express human CD14 and endogenous (hamster) TLR4 and MD-2 respond to lipid IVa and RsDPLA by up-regulating the expression of CD25 and producing IL-6, while over-expression of human TLR4 abolishes these responses. Conversely, over-expression of hamster TLR4 in human THP-1 cells, which express endogenous (human) CD14, TLR4 and MD-2, renders the cells responsive to lipid IVa and RsDPLA, while over-expression of human TLR4 merely increases the response to enteric LPS as compared to untransfected cells. In contrast to these findings, transfection experiments in HEK 293 cells using human and murine receptor proteins suggest that MD-2 and not TLR4 is responsible for the species-specific response to lipid IVa, and in transfected Ba/F3 cells, human MD-2 confers on murine TLR4 the ability to recognize lipid IVa as an antagonist. Unfortunately, the reverse experiment, i.e. co-expression
of human TLR4 and murine MD-2, did not yield a functional receptor in the latter study. MD-2 may regulate ligand recognition by determining whether or not the conformation of TLR4 changes in response to a stimulus, thereby permitting oligomerization and signal transduction to occur.\textsuperscript{125} Another alternative, however, is that both TLR4 and MD-2 contribute to the species-specific response. This hypothesis is supported by transfection experiments in human THP-1 cells using human or murine CD14, TLR4 and MD-2, in which the cellular response to heptaacylated \textit{Salmonella} lipid A was determined.\textsuperscript{248} Over-expression of human receptor proteins increased the cells' responsiveness to lipid A from \textit{E. coli}, but did not confer responsiveness to \textit{Salmonella} lipid A. Expression of murine receptor proteins, on the other hand, rendered cells responsive to both types of lipid A.\textsuperscript{248} Neither receptor combination, i.e. human TLR4 with murine MD-2, or murine TLR4 with human MD-2, recognized \textit{Salmonella} lipid A as an agonist, which suggests that both proteins participate in ligand recognition. In an attempt to unify these findings, it was proposed that TLR4 and MD-2 have to be expressed together to form a functional lipid A recognition site.\textsuperscript{100} The dependence of biological responses on TLR4 and/or MD-2 may then depend upon the relative expression levels of the two proteins.\textsuperscript{100}

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CHAPTER 3
LIPOPOLYSACCHARIDE FROM RHODOBACTER SPHAEROIDES IS AN AGONIST
IN EQUINE CELLS

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ABSTRACT

Endotoxemia is associated with the principle causes of death in adult horses and equine neonates and, therefore, veterinary researchers are expending efforts to identify new therapeutic interventions that might be beneficial in these animals. Endotoxin antagonists inhibit interaction of endotoxin with cellular receptors and may be beneficial in the treatment of endotoxemia and sepsis. Diphosphoryl lipid A from Rhodobacter sphaeroides (RsDPLA) is a potent antagonist of enteric LPS in human cells, but is an agonist in hamster cells. In this study, the effect of lipopolysaccharide from R. sphaeroides (RsLPS) on equine whole blood and isolated monocyte preparations was investigated by comparing tumor necrosis factor (TNF) production in response to RsLPS and Escherichia coli O55:B5 LPS. Our results indicate that RsLPS is a potent agonist in equine cells, which precludes therapeutic use of this agent in equine patients. In contrast to the results in equine cells, RsLPS did not elicit TNF production by itself, and inhibited the response to E. coli O55:B5 LPS in a human monocytic cell line.

INTRODUCTION

Endotoxemia is an important clinical problem in horses and is implicated in the pathogenesis of conditions such as acute gastrointestinal disease and Gram-negative neonatal sepsis.\(^1,2\) Endotoxemia has been associated with the development of life-threatening complications, such as laminitis,\(^3\) disseminated intravascular coagulopathy\(^4\) and organ failure;\(^5\) it is also responsible for major economic losses due to treatment cost, long-term complications and death. Exploration of new treatment options is, therefore, desirable.
Endotoxin (lipopolysaccharide, LPS) is a structural component of the outer cell membrane of Gram-negative bacteria, which is released during rapid proliferation and in the event of bacterial cell death.\textsuperscript{6} LPS molecules consist of a repeating polysaccharide chain (O-antigen), a non-repeating oligosaccharide core region and the lipid A portion.\textsuperscript{7} Lipid A is the biologically active part of LPS\textsuperscript{8,9} and its structure is highly conserved among different groups of Gram-negative enteric bacteria, such as \textit{Escherichia coli} or \textit{Salmonella spp.}\textsuperscript{10} Lipid A of \textit{E. coli} consists of a 1,4'-bis-phosphorylated $\beta$-1,6-linked D-glucosamine disaccharide backbone substituted with six fatty acyl chains.\textsuperscript{7} The distribution of fatty acyl chains in the lipid A molecule is asymmetric.

Activation of inflammatory cells, primarily mononuclear phagocytes, occurs after LPS forms high-affinity complexes with lipopolysaccharide-binding protein (LBP),\textsuperscript{11} and interacts with pattern recognition receptors on the cell surface. Essential components of the LPS-receptor complex have been identified as cluster differentiation antigen 14 (CD14) and Toll-like receptor 4 (TLR4) in association with MD-2.\textsuperscript{12-14} An important consequence of cell activation is the increased production of cytokines, which mediate many of endotoxin's pathophysiological effects. Inhibition of LPS-receptor interaction with endotoxin antagonists, therefore, represents a potential avenue of treatment.

Endotoxin analogues, which are similar in structure to enteric LPS or lipid A, but have reduced or no toxicity may act as LPS antagonists.\textsuperscript{15} Some of the most promising analogues evaluated to date are diphosphoryl lipid A from \textit{Rhodobacter sphaeroides} (RsDPLA), and E5531, a synthetic compound based on the \textit{Rhodobacter capsulatus} structure. RsDPLA, which cross-reacts serologically with enteric lipid A,\textsuperscript{16} has the same backbone as enteric lipid A, but differs in the type and distribution of its fatty acyl chains. In human and murine cells, purified
RsDPLA inhibited cellular binding of enteric LPS, cytokine release in response to cell stimulation by enteric LPS, and expression of LPS-inducible genes. Furthermore, decreased lethality after challenge with LPS has been documented in mice administered purified RsDPLA. The mechanism of antagonism is thought to be primarily competitive, with targets for competition including LBP, CD14, TLR4 or other receptor components. In contrast to the antagonism observed in human and murine systems, synthetic RsDPLA is an agonist in hamster cells. Transfection studies showed that TLR4 and not CD14 determined the phenotype of cellular response to RsDPLA in these species. The three-dimensional structure of TLR4 is unknown at this time and the structural basis for agonistic or antagonistic responses to RsLPS and RsDPLA remains elusive.

The objective of this study was to evaluate the response of equine whole blood and peripheral blood monocytes to RsLPS in order to determine the therapeutic potential of this compound in horses. RsLPS rather than RsDPLA was used in the study because a smooth LPS from E. coli O55:B5 was used as the positive control stimulus. To ascertain that the RsLPS possessed similar biologic activity as RsDPLA used in previous studies, the response of equine cells was compared to that of Mono Mac 6 cells, a human monocytic cell line.

MATERIALS AND METHODS

Reagents: LPS from E. coli O55:B5 and RsLPS were purchased from List Biologicals (Campbell, CA, USA). RPMI-1640 with L-glutamine was from Bio Whittaker (Walkersville, MD, USA). Penicillin-Streptomycin solution (PS; 10,000 units/ml penicillin, 10 mg/ml streptomycin), phosphate buffered saline pH 7.4 (PBS), OPI media supplement (0.15 g oxaloacetate, 0.05 g pyruvate, 0.0082 g bovine insulin), and 1α,25-dihydroxy-vitamin D₃, were
from Sigma Chemical Co. (St. Louis, MO, USA). Fetal bovine serum (FBS, defined, 40 nm filtered) was from HyClone Laboratories Inc. (Logan, UT, USA). Heparin (Hep-Lock, 10 units/ml) was from Elkins-Sinn, Inc. (Cherry Hill, NJ, USA).

**Horses:** Twelve adult horses were used for this study. Horses were determined to be healthy based on clinical examination. Blood was taken from the jugular vein after sterile preparation of the venipuncture site. The Animal Care and Use Committee at the University of Georgia approved the experimental protocol.

**LPS preparation:** LPS was reconstituted in phosphate buffered saline at a concentration of 1 mg/ml. Prior to use, LPS was sonicated for 30 s and further diluted in RPMI-1640.

**Whole blood stimulation:** Heparinized whole blood (10 units heparin/ml) was divided into 950 µl aliquots and placed into sterile 1 ml polypropylene cryovials. LPS (in 50 µl) was added to achieve final concentrations of 0, 0.1, 0.32, 1, 3.2 and 10 ng/ml blood. Samples were incubated on an orbital mixer at 37°C for 6 hours, and at the end of incubation, plasma was collected by centrifugation and stored frozen until assayed for TNF activity.

**Monocyte stimulation:** Mononuclear cells were isolated by density gradient centrifugation as previously described. Aliquots of 1 ml (3x10⁶ cells) were added to sterile 12x75 mm polystyrene tubes and incubated for 2 hours (37°C, 5% CO₂ in air), after which time non-adherent cells were removed by 3 washes with PBS. Although not performed in this study, non-specific esterase staining has previously been used in our laboratory to demonstrate that > 85% of adherent cells isolated in this fashion are monocytes. Monocytes were overlaid with 1 ml of culture medium (RPMI-1640 with 1% PS solution and 5% FBS) containing LPS at concentrations of 0, 0.01, 0.1, 1, 10 and 100 ng/ml. Cells were incubated for 6 hours (37°C, 5% CO₂ in air), after which cell media were collected for TNF assay.
**Mono Mac 6 cell stimulation:** Mono Mac 6 cells were provided by Dr. H.W.L. Ziegler-Heitbrock (University of Munich, Germany). Cells were grown in RPMI-1640 with 1% PS, 10% FBS and 1% OPI supplement. For 48 hours prior to incubation with LPS, cells were treated with vitamin D (0.01 µg/ml cells) to allow differentiation and enhance expression of CD14. Immediately prior to experiments, cells were pelleted by centrifugation and suspended in RPMI-1640 containing 1% PS and 10% FBS. Aliquots (950 µl; 1x10^6 cells) were added to 12x75 mm polypropylene tubes and LPS (in 50 µl) was added to achieve final concentrations of 0, 0.01, 0.1, 1, 10 and 100 ng/ml. Cells were incubated for 6 hours (37°C, 5% CO2 in air), after which time cell media were collected for TNF assay. For antagonism experiments, cells were pre-incubated with RsLPS at final concentrations of 1, 10, 100, 1000 and 10,000 ng/ml for 1 h and subsequently stimulated with *E. coli* O55:B5 LPS (10 ng/ml final concentration) for 6 h.

**TNF assays:** TNF activity in equine samples was determined using an *in vitro* cytotoxicity bioassay as described previously. The TNF concentration in samples obtained from Mono Mac 6 cells was determined using a solid-phase sandwich ELISA as described previously.

**Data analysis and statistics:** LPS concentration-response data were analyzed by fitting a logistic expression to these results using the GraphPad Prism software. This analysis allowed determination of EC50 and maximum response values with associated 95% confidence intervals (CI), which were used to discern significant differences between the response to *E. coli* O55:B5 LPS and RsLPS.
RESULTS

**RsLPS is an agonist in equine cells**

To assess the effect of RsLPS on equine cells, whole blood and isolated monocyte preparations were exposed to increasing concentrations of the compound, with *E. coli* O55:B5 LPS used as a control stimulus. For each preparation the experiments were repeated 8 times in different horses. Average TNF activities measured in response to RsLPS and *E. coli* O55:B5 LPS are presented in Figure 3.1 (whole blood) and Figure 3.2 (monocytes).

The average maximal TNF production in response to RsLPS was 300.0 units/ml (95% CI: 81.1 - 447.6 units/ml) in whole blood and 288.2 units/ml (95% CI: 201.4 - 375.2 units/ml) in monocytes. This response did not differ significantly from that observed in response to *E. coli* O55:B5 LPS, where an average maximal TNF response of 598.0 units/ml (95% CI: 139.4 - 1057.0 units/ml) in whole blood and 369.7 units/ml (95% CI: 242.4 - 496.9 units/ml) in monocytes was observed. Similarly, values for the EC\(_{50}\) (*i.e.* the concentration of LPS eliciting a half-maximal response) of RsLPS and *E. coli* O55:B5 LPS did not differ significantly in either whole blood or monocytes. For whole blood, the average RsLPS EC\(_{50}\) was 1.48 ng/ml (95% CI: 0.16 - 13.24 ng/ml), while the *E. coli* O55:B5 LPS EC\(_{50}\) was 2.38 ng/ml (95% CI: 0.28 - 20.14 ng/ml). For monocytes, the average RsLPS EC\(_{50}\) was 0.05 ng/ml (95% CI: 0.006 - 0.39 ng/ml) and the *E. coli* O55:B5 LPS EC\(_{50}\) was 0.03 ng/ml (95% CI: 0.002 - 0.33 ng/ml). These data show that RsLPS is a potent stimulus for TNF production in equine cells.

**RsLPS is an antagonist in Mono Mac 6 cells**

We observed stimulation of TNF production in equine cells exposed to RsLPS that was similar in magnitude to that elicited by *E. coli* O55:B5 LPS. To test our RsLPS compound for
potential contamination, alteration by storage or inherently different activity than that of the
more commonly used RsDPLA, we tested the compound in a human monocytic cell line (Mono
Mac 6). In two independent experiments, strong stimulation of Mono Mac 6 cells in response to
*E. coli* O55:B5 LPS was observed (average maximal TNF production was 1505 pg/ml), while
TNF production in response to RsLPS did not differ significantly from baseline values (data not
shown). We further showed that RsLPS did indeed act as an endotoxin antagonist in the Mono
Mac 6 cells. At a concentration of 100 ng/ml, RsLPS completely inhibited TNF production in
response to a 10 ng/ml stimulus of *E. coli* O55:B5 LPS (Figure 3.3). Our findings, therefore,
suggest a species-specific difference in the response to RsLPS by equine cells.

**DISCUSSION**

The results of this study indicate that RsLPS acts as a potent agonist in equine cells. The
agonistic effect was likely due to direct stimulation of monocytes, as TNF production was
induced in whole blood as well as isolated monocyte preparations. From the results of this study,
we conclude that LPS antagonists based on the structure of RsLPS should not be used in horses.

We used 95% confidence intervals to compare responses to *E. coli* O55:B5 LPS and
RsLPS. For both types of equine cell preparations, the 95% confidence intervals for the maximal
response to and the EC$_{50}$ of *E. coli* O55:B5 LPS and RsLPS overlapped, indicating that there was
no significant difference in the response to the two compounds. Because this approach only
evaluated average values of the 8 experiments, and there is remarkable variation in the TNF
response among horses, we also compared responses to *E. coli* O55:B5 LPS versus RsLPS for
each individual animal. Using whole blood assays, 3/8 horses showed a similar response to both
compounds while 5/8 responded more strongly to *E. coli* O55:B5 LPS. Using monocyte
preparations, 4/8 horses responded similarly, 3/8 responded more strongly to *E. coli* O55:B5 LPS and 1/8 responded more strongly to *Rs*LPS. Therefore, although not of statistical significance, there was a trend for *Rs*LPS to be less potent than *E. coli* O55:B5 LPS in stimulating equine cells.

Because the majority of previously published studies concerning the biological activity of LPS compounds derived from *R. sphaeroides* used DPLA (*Rs*DPLA) and not LPS, we performed control experiments in Mono Mac 6 cells to ascertain that the LPS compound showed the expected antagonistic activity. Another concern was the chemical stability of the LPS compound and the potential for generation of biologically active breakdown products over time. In studies utilizing *Rhodobacter capsulatus* lipid A, for example, a structurally similar compound to *Rs*DPLA, generation of side products with weak agonistic activity in murine cells was observed upon storage of the compound for extended periods of time. Our control experiments showed no significant activation of Mono Mac 6 cells by *Rs*LPS. In addition, *Rs*LPS completely antagonized the response to enteric LPS. It is, therefore, highly unlikely that the observed activation of equine cells was the result of degradation of the LPS compound, and we conclude that the response of equine cells to *Rs*LPS observed in this study represents a species-specific characteristic. Although it would further confirm our findings to evaluate the effect of *Rs*DPLA in equine cells as well, we have no reason to expect that the *Rs*DPLA would behave differently from *Rs*LPS.

Differences in the TLR4 receptor are purportedly responsible for phenotypic variation in the response to *Rs*DPLA between hamster cells, murine and human cells. In a recent report by Hajjar et al., the hypervariable region of the middle domain of TLR4 was determined to be responsible for differential recognition of penta-acylated LPS from *Pseudomonas aeruginosa* by
human and murine cells. Alignment of TLR4 protein sequences including that of equine TLR4 in their report demonstrates that hypervariability of this region also exists between equine and human TLR4. We, therefore speculate that differences in TLR4, presumably in the hypervariable region of the middle domain, may be responsible for the species-specific response of equine cells to RsLPS observed in our study. Investigation of differences between equine and human MD-2 and their effect on the cellular response to RsLPS and RsDPLA is further warranted because MD-2 was found to be responsible for species-specific recognition of the lipid IVa$^{28}$ and *Salmonella* lipid A.$^{29}$

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Figure 3.1: Tumor necrosis factor (TNF) production by equine whole blood in response to LPS from *E. coli* O55:B5 and *R. sphaeroides*. For RsLPS, average maximal TNF production was 300.0 units/ml (95% CI, 81.1 - 447.6 units/ml) and EC$_{50}$ was 1.48 ng/ml (95% CI, 0.16 - 13.24 ng/ml). For *E. coli* O55:B5 LPS, average maximal TNF production was 598.0 units/ml (95% CI, 139.4 - 1057.0 units/ml) and EC$_{50}$ was 2.38 ng/ml (95% CI, 0.28 - 20.14 ng/ml). Maximal TNF response and EC$_{50}$ did not differ significantly for the two compounds. The results shown represent data obtained from 8 independent experiments.
Figure 3.2: Tumor necrosis factor (TNF) production by equine isolated monocytes in response to LPS from *E. coli* O55:B5 and *R. sphaeroides*. For RsLPS, average maximal TNF production was 288.2 units/ml (95% CI, 201.4 - 375.2 units/ml) and EC$_{50}$ was 0.05 ng/ml (95% CI, 0.006 - 0.39 ng/ml). For *E. coli* O55:B5 LPS, average maximal TNF production was 369.7 units/ml (95% CI, 242.4 - 496.9 units/ml) and EC$_{50}$ was 0.03 ng/ml (95% CI, 0.002 - 0.33 ng/ml).

Maximal TNF response and EC$_{50}$ did not differ significantly for the two compounds. The results shown represent data obtained from 8 independent experiments.
**Figure 3.3:** RsLPS inhibits TNF production by Mono Mac 6 cells stimulated with *E. coli* O55:B5 LPS. Cells were pre-incubated for 1 hour with RsLPS at final concentrations of 1, 10, 100, 1,000 and 10,000 ng/ml and were subsequently stimulated with 10 ng/ml of *E. coli* O55:B5 LPS for 6 hours.
CHAPTER 4

THE EQUINE TLR4/MD-2 COMPLEX MEDIATES RECOGNITION OF LPS FROM

RHODOBACTER SPHAEROIDES AS AN AGONIST

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ABSTRACT

Lipopolysaccharide (LPS) antagonists are LPS molecules of atypical structure, which inhibit the response of inflammatory cells to endotoxin and may be of therapeutic value in the treatment of endotoxemia and sepsis. The antagonistic effect of some LPS compounds is restricted to certain host species, while others recognize them as agonists with endotoxic activity. TLR4 and/or MD-2 are components of the LPS receptor complex that likely determine recognition of LPS compounds as agonists or antagonists. Lipid A from Rhodobacter sphaeroides (RsLA) is a potent LPS antagonist in human and murine cells, but is an agonist in hamster cells. LPS from Rhodobacter sphaeroides (RsLPS) is an agonist in equine cells. To determine the role of equine receptor proteins in the recognition of RsLPS as an agonist, transfection experiments in HEK293 cells were performed. Cells were transfected with human or equine CD14, TLR4 and MD-2, or with combinations of equine and human receptors. NF-κB activation was measured as an indicator of cellular activation. RsLPS activated NF-κB in cells expressing equine receptor proteins but not in those expressing human receptor proteins. Activation of NF-κB in cells expressing equine receptor proteins occurred independent of CD14. All possible combinations of equine and human receptor proteins yielded functional receptor complexes for enteric (E. coli) LPS, while RsLPS stimulated NF-κB activation only in cells expressing both TLR4 and MD-2 of equine origin. We conclude that RsLPS-mediated stimulation of equine cells is dependent on TLR4 and MD-2. RsLPS may be able to stimulate equine cells independent of CD14.
INTRODUCTION

The response to endotoxin (lipopolysaccharide, LPS) is crucial for the innate immune response to Gram negative bacteria. However, cellular responses to endotoxin also contribute to the development of disease if they are activated in an exaggerated or uncontrolled fashion. In horses, endotoxemia is a frequent and serious complication of acute gastrointestinal disturbances (colic) as well as septic processes, has a guarded prognosis, and may have serious, long term effects. Elucidation of the mechanisms of endotoxin interaction with cells is, therefore, an important step in the development of novel treatment strategies.

Endotoxin is an integral component of the outer membrane of the Gram-negative bacterial cell wall, and is released upon bacterial replication as well as bacterial cell death. Endotoxin molecules, through their biologically active lipid A portion, interact with a cellular receptor complex consisting of the cluster differentiation antigen CD14, Toll-like receptor 4 (TLR4) and MD-2. In response to ligand-receptor interaction, intracellular signaling pathways are activated, and cellular functions are altered, which is referred to as cellular activation. Cellular activation results in the activation and translocation of transcription factors such as NF-kB, which allow directed up-regulation of inflammatory gene expression to occur. An important consequence of cellular activation is the production of a multitude of cytokines and other inflammatory mediators, which mediate many of the pathophysiological effects of endotoxin.

LPS antagonists are molecules of lipopolysaccharide structure, which do not activate inflammatory cells but instead inhibit the cellular response to endotoxin. The mechanism of inhibition is thought to be competitive, however, additional mechanisms such as the release of anti-inflammatory glucocorticoids have been proposed. Aside from providing new treatment
approaches for endotoxemia and sepsis, LPS antagonists serve as useful research tools to investigate ligand-receptor interactions and structure-function relationships of LPS. It has been determined that species-specific differences exist in the cellular response to some LPS antagonists, such that these molecules block the response to endotoxin in some species but act as agonists themselves in others. For example, lipid IVa is an antagonist in human cells but an agonist in murine and hamster cells. Similarly, LPS (RsLPS) and lipid A (RsLA) from Rhodobacter sphaeroides are antagonists in human and murine cells, but agonists in hamster cells. Based on the results of transfection studies, species-specific recognition of LPS antagonist has been attributed to TLR4, MD-2 or the TLR4/MD-2 complex. We have previously demonstrated that RsLPS potently stimulates the production of tumor necrosis factor (TNFα) in isolated equine monocytes, while it inhibits TNFα production in response to enteric LPS in human monocyctic cells. Because of this striking difference in the response of human and equine cells to RsLPS, we sought to determine which receptor protein was responsible for recognition of RsLPS as an agonist in equine cells. Consequently, we expressed human and equine receptor proteins in HEK293 cells, and determined that RsLPS caused NF-κB activation in cells expressing equine but not human receptor proteins, and that the response was mediated by equine TLR4 and MD2 independent of CD14. By combining equine and human proteins, we further determined that both TLR4 and MD-2 need to be of equine origin to elicit a response to RsLPS.

MATERIALS AND METHODS

Lipopolysaccharides: LPS from E. coli O55:B5 and RsLPS were purchased from List Biologicals, Campbell, CA.
**Plasmids:** Human CD14 in pcDNA 3.1, human MD-2 in pEF-BOS and the firefly luciferase reporter plasmid pELAM were a generous gift from D. Golenbock, Worcester, MA. Human TLR4 in pEF-BOS was a generous gift from K. Miyake, Tokyo, Japan. The renilla luciferase reporter plasmid pTKRL was purchased from Promega Corp., Madison, WI. Equine CD14 was cloned into pcDNA 3.1 (Invitrogen, Carlsbad, CA) in our laboratory. Equine TLR4 and equine MD-2 were cloned into pBluescript (Stratagene, La Jolla, CA) in our laboratory. For subcloning of equine TLR4 and equine MD-2 into pEF-BOS, inserts were produced by PCR using the Advantage™-HF2 PCR kit (BD Biosciences Clontech, Palo Alto, CA). PCR primers were used that introduced *XhoI* and *BamHI* restriction sites at the 5' and 3' ends of the inserts, respectively. For TLR4, the forward and reverse primer sequences were (5'→3') : 5'-gatctcagcttctggcagcagaaatgc-3' and (3'→5') : 5'-gatggatccggttctgc-3'. For MD-2, the primer sequences were (5'→3') : 5'-gatctcagttctggcagcagaaatgc-3' and (3'→5') : 5'-gatggatccggttctgc-3'. Inserts were digested with *XhoI* and *BamHI* (Promega Corp., Madison, WI), submitted to gel electrophoresis and purified from the gel using the QIAquick® gel extraction kit (Qiagen, Valencia, CA). pEF-BOS plasmid containing human TLR4 was digested with *XhoI* and *BamHI*, and vector separated from insert by gel electrophoresis and purification. New inserts were ligated into the vector using the TaKaRa DNA ligation kit, ver.2 (TAK6022, Takara Mirus Bio, Kyoto, Japan and Madison, WI). Ligation reactions were purified using the QIAquick® PCR purification kit (Qiagen, Valencia, CA), and Top10 competent *E. coli* (Invitrogen, Carlsbad, CA) were transformed using electroporation. Plasmids were purified using the QIAprep® miniprep kit and EndoFree® plasmid maxi kit (Qiagen, Valencia, CA). Correctly ligated plasmids were confirmed using restriction digestion and sequencing techniques.
**RNA isolation and RT-PCR:** HEK293 cells were obtained from American Type Culture Collection (ATCC), Manassas, VA. HEK293 cells were maintained in Minimum Essential Medium (MEM) with penicillin/streptomycin and 10% fetal bovine serum (FBS). Cells were grown in T25 cell culture flasks to approximately 50-60% confluency and were transfected with equine or human receptor protein plasmids (CD14, TLR4 and MD-2; 0.5 µg/plasmid) using PolyFect® reagent (Qiagen, Valencia, CA) at a ratio of 15 µl PolyFect®/µg plasmid DNA. Fresh tissue culture medium was applied 6 hours after transfection, and cells were maintained for approximately 36 hours before RNA isolation. RNA was isolated using the Absolutely RNA kit (Stratagene, La Jolla, CA). RNA was submitted to restriction digestion using AluI (Promega Corp., Madison, WI) in the presence of RNase inhibitor (RNAsin, Promega Corp., Madison, WI) to destroy remaining plasmid DNA. RNA was used as a template for reverse transcription and polymerase chain reaction (RT-PCR) using the One-Step RT-PCR kit from Qiagen (Qiagen, Valencia, CA). Primers used to amplify fragments were: for human CD14: (5’→3’) 5’-aagccttccagtgtctctgc-3’ and (3’→5’) 5’-ttcgcaggtcaggttg-3’; for human TLR4: (5’→3’) 5’-tcattttccctggtgagtgtgac-3’ and (3’→5’) 5’-ggacaagcattgaagatgcc-3’; for human MD-2: (5’→3’) 5’-gaagctcagaagcagtattgggtc-3’ and (3’→5’) 5’-ggtgtaggatgacaaactccaagc-3’; for equine CD14: (5’→3’) 5’-gtgcactcactgccttttcc-3’ and (3’→5’) 5’-cctgtttcaaagtggaatgctgg-3’; for equine TLR4: (5’→3’) 5’-tcactcactcaggtgatgac-3’ and (3’→5’) 5’-ggacaagcattgaagatgcc-3’; for equine MD-2: (5’→3’) 5’-caatgcgtaaagaggttttcc-3’ and (3’→5’) 5’-ggtgtaggatgacaaactccaagc-3’. Control PCR reactions not including a reverse transcription step were performed with the same primers and templates using the MasterTaq kit (Eppendorf, Hamburg, Germany). RNA isolated from human Mono Mac 6 cells and equine monocytes served as positive control templates, and RNA isolated
from HEK293 cells served as a negative control template. For detection, PCR products were submitted to gel electrophoresis on a 1.5% agarose gel.

**HEK293 transfection and stimulation:** HEK293 cells were seeded in 96-well polystyrene tissue culture plates at approximately 20,000 cells/well to achieve a density of 60-80% at the time of transfection. The following day, cells were transfected with receptor plasmids (1 ng/well) using PolyFect® reagent at a ratio of 1.5 µl PolyFect®/100 ng plasmid DNA. In the results section, the type and species origin of plasmids used for transfection are indicated; e.g., hCD14/hTLR4/eMD-2 denotes cells transfected with CD14 and TLR4 of human origin and MD-2 of equine origin. The cells also were transfected with two luciferase reporter plasmids: pELAM, encoding an NF-κB inducible firefly luciferase (50 ng/well), and pTKRL, encoding a constitutively active renilla luciferase (5 ng/well). Total DNA in the transfections was adjusted to 100 ng/well using empty pcDNA3.1 plasmid. Following transfection for 6 hours, fresh tissue culture medium was added and cells were maintained for 36-42 hours prior to stimulation with LPS. Cells were washed once in serum-free medium prior to addition of LPS. LPS was diluted in tissue culture medium containing 0.5% FBS, incubated for 15-30 min at room temperature, diluted 20-fold in serum free medium and added to cells. The final concentration of FBS in the assays was therefore 0.025%. Recombinant human TNFα (10 ng/ml) was used as a control stimulus to assess luciferase reporter function. Cells were incubated for 4 hours, then washed three times in phosphate buffered saline and lysed in passive lysis buffer (50 µl/well) provided with the luciferase reporter assay system kit. 20 µl of cell lysate/well was transferred to a 96-well plate suitable for luminescence reading and luciferase activity determined using a dual-luciferase® reporter assay system (Promega Corp., Madison, WI) and a Fluoroskan Ascent FL plate luminometer (Thermo Labsystems). Individual experiments were performed in duplicate or
triplicate with treatments assigned randomly to the wells in a 96-well plate; the number of repetitions of an experiment (n) is indicated in the results section.

**Data analysis:** For each sample, the firefly luciferase value was divided by the renilla luciferase value and the ratio reported in relative light units (RLU). Baseline RLU were determined from measurements of luciferase activity in unstimulated cells. Data were analyzed using analysis of variance in a randomized block design to account for an effect of experiment repetitions on different days. RLU of LPS-stimulated cells were compared to those of unstimulated cells using the least square means. Comparisons were only performed within groups of cells expressing the same receptor proteins, as baseline values differed depending on the transfection status of cells. Differences were considered significant at \( p \leq 0.05 \). In the graphs, fold-increases of RLU over baseline (FI\textsubscript{BL}) are reported, which were calculated by dividing RLU of LPS-stimulated cells by baseline RLU.

**RESULTS**

*RsLPS stimulates NF-κB activation in HEK293 cells expressing equine CD14, TLR4 and MD-2, but not in cells expressing human receptor proteins*

HEK293 cells were transfected with plasmids encoding equine or human CD14, TLR4, and MD-2, and production of mRNA was demonstrated by RT-PCR (Figure 4.1). Because plasmid contamination of RNA was identified initially, RNA samples were submitted to restriction digestion with AluI, and PCR without reverse transcription performed to ensure the absence of plasmid DNA (data not shown). RT-PCR using RNA from non-transfected HEK293 cells as the template showed minor unspecific amplification in the absence of specific bands.
HEK293 cells expressing equine or human receptor proteins were stimulated with *E. coli* LPS or RsLPS. Cells transfected only with the reporter plasmids were used as a control to identify receptor-independent effects of LPS. HEK293 cells expressing equine CD14, TLR4 and MD-2 responded to RsLPS by NF-κB activation, which was comparable in magnitude with that induced by *E. coli* LPS (Figure 4.2). Fold-increase of RLU over baseline was 19.87 ± 7.77 in response to *E. coli* LPS (10 ng/ml, n=9, p<0.0001), 11.98 ± 6.23 in response to RsLPS (10 ng/ml, n=9, p<0.0001) and 13.84 ± 6.97 in response to RsLPS (100 ng/ml, n=2, p=0.0003). In contrast, HEK293 cells transfected with human receptor plasmids did not respond to RsLPS by NF-κB activation. Fold increase of RLU over baseline was 28.97 ± 20.78 in response to *E. coli* LPS (10 ng/ml, n=8, p<0.0001), 1.45 ± 1.19 in response to RsLPS (10 ng/ml, n=7, p=0.41) and 2.43 ± 1.79 in response to RsLPS (100 ng/ml, n=4, p=0.48). At a concentration of 1 µg/ml, RsLPS resulted in slight and inconsistent activation of NF-κB in cells expressing human receptor proteins (FI_BL = 4.52 and 1.82, respectively, n=2, data not shown). Cells transfected only with reporter plasmids did not exhibit NF-κB activation in response to *E. coli* LPS at a concentration of 10 ng/ml (FI_BL = 1.01 ± 0.24, n=4, p=0.12) or RsLPS at a concentration of 100 ng/ml (FI_BL = 1.14 ± 0.2, n=2, p=0.3).

**HEK293 cells expressing equine TLR4/MD-2 but not CD14 respond to RsLPS**

To determine the contribution of each receptor protein to the cellular response to RsLPS, HEK293 cells were transfected with equine CD14, TLR4 or MD-2 individually, each combination of 2 plasmids, and the combination of all 3 plasmids. Cells were exposed to *E. coli* LPS or RsLPS (10 ng/ml) and fold-increase of RLU determined (Figure 4.3). For *E. coli* LPS, the response was strongest when all 3 proteins were expressed concurrently (FI_BL = 14.59 ± 9.17,
n=6, p<0.0001); compared to baseline, RLU did not increase significantly in cells expressing individual receptor proteins or combinations of two proteins. Cells transfected with TLR4 and MD-2 but not CD14 that were stimulated with *E. coli* LPS in the presence of 10% FBS responded as strongly as cells transfected with all three receptors and stimulated in the presence of 0.025% FBS (data not shown). In response to RsLPS, RLU increased significantly in cells expressing all 3 proteins (FIBL= 9.54 ± 5.46, n=3, p=0.0004) and cells expressing TLR4 and MD-2 but not CD14 (FIBL= 10.79 ± 6.92, n=3, p=0.001). RLU did not increase significantly in cells expressing only CD14, only TLR4, only MD-2, CD14 and TLR4, or CD14 and MD-2.

**In HEK293 cells expressing human receptor proteins, RsLPS inhibits NF-κB activation in response to *E. coli* LPS**

Because *Rs*LPS inhibits TNFα production by human monocytic cells stimulated with *E. coli* LPS, we investigated whether it would also inhibit *E. coli* LPS-induced NF-κB activation. HEK293 cells transfected with human CD14, TLR4 and MD-2 were incubated with RsLPS (10 ng/ml to 1 µg/ml) for one hour, and then stimulated with *E. coli* LPS (10 ng/ml) for an additional 4 hours (Figure 4.4). Cells incubated with media alone were used to determine baseline RLU and cells stimulated with *E. coli* LPS in the absence of RsLPS served as the positive control. RsLPS inhibited NF-κB activation in a dose-dependent manner with complete inhibition (RLU not significantly different from baseline) occurring at a concentration of RsLPS of 1 µg/ml.
The species origin of both TLR4 and MD-2 determines the response of transfected HEK293 cells to RsLPS

To investigate the contribution of individual receptor proteins to the equine-specific response to RsLPS, HEK293 cells were transfected with CD14, TLR4 and MD-2 using all possible combinations of human and equine receptor plasmids, and stimulated with *E. coli* LPS and RsLPS (10 ng/ml). Control cells were transfected with human or equine plasmids alone, and the response of cells to *E. coli* LPS was used to assess the functionality of receptor complexes consisting of human and equine plasmids. Although there was variation in the magnitude of response, all combinations of human and equine proteins yielded functional receptors as shown by their significant response to *E. coli* LPS (Figure 4.5 and 4.6). In 3 experiments, fold-increase of RLU over baseline in response to *E. coli* LPS ranged from 13.93 ± 4.83 (hCD14/eTLR4/hMD-2, p=0.036) to 37.06 ± 6.99 (eCD14/hTLR4/hMD-2, p<0.0001). Cells expressing only human receptors had a fold-increase of RLU over baseline of 25.94 ± 10.95 (p<0.0001), and those expressing only equine receptors had a fold-increase of RLU over baseline of 24.8 ± 4.31 (p<0.0001).

No difference in response was identified between cells expressing only human receptor proteins and those expressing human TLR4/MD-2 and equine CD14 on the one hand, and cells expressing only equine receptor proteins and those expressing equine TLR4/MD-2 and human CD14, on the other hand (Figure 4.5). In response to 10 ng/ml of RsLPS (n=3), fold-increases of RLU over baseline were 1.23 ± 0.68 (p=0.66) for hCD14/hTLR4/hMD-2, 1.32 ± 0.6 (p=0.45) for eCD14/hTLR4/hMD-2, 14.49 ± 4.72 (p<0.0001) for eCD14/eTLR4/eMD-2 and 11.85 ± 4.71 (p<0.0001) for hCD14/eTLR4/eMD-2. Increasing the concentration of RsLPS to 100 ng/ml yielded similar results.
RsLPS stimulated NF-κB activation only in cells expressing both equine TLR4 and MD-2, and responses were similar at concentrations of RsLPS of 10 and 100 ng/ml (Figure 4.6). In response to 10 ng/ml of RsLPS (n=3), average fold-increases of RLU over baseline were 1.07 ± 0.27 (hCD14/eTLR4/hMD-2, p=0.99), 1.06 ± 0.24 (hCD14/hTLR4/eMD-2, p=0.89), 2.19 ± 0.95 (eCD14/hTLR4/eMD-2, p=0.14) and 1.18 ± 0.09 (eCD14/eTLR4/hMD-2, p=0.87).

**DISCUSSION**

Mammalian species exhibit considerable variation in their sensitivity to endotoxin. For example, humans and ungulates are highly sensitive, while mice and rats respond much less strongly. In addition to this variability in the sensitivity to endotoxin, qualitative differences in the response to certain LPS compounds have been described. Structurally unique LPS compounds that differ from enteric LPS appear to have individual species patterns concerning their agonistic or antagonistic effects. Because structural features of LPS receptor proteins are likely responsible for the recognition of a compound as an agonist, understanding the nature of ligand-receptor interactions is required to predict the effects of a particular LPS compound in a species of interest. Knowledge of these interactions may also facilitate the design of species-specific LPS antagonists as a treatment for endotoxemia and sepsis.

In equine cells, RsLPS is a potent agonist eliciting TNFα production by monocytes with equal potency and efficacy as enteric LPS from *E. coli*. In human and murine cells, on the other hand, RsLPS and RsLA are endotoxin antagonists. RsLPS and RsLA are, therefore, useful in identifying equine-specific ligand-receptor interactions. In this study, we sought to identify the
equine receptor protein that is responsible for the recognition of RsLPS as an agonist with endotoxic activity. Using a dual luciferase reporter assay in transfected HEK293 cells to measure NF-κB activation in response to LPS, we determined that RsLPS did not stimulate NF-κB activation in cells expressing human receptor proteins, while it was a potent agonist in cells expressing equine receptor proteins (Figure 4.2). To our knowledge, this is the first report of successful expression of equine receptor proteins in HEK293 cells. RsLPS inhibited the cellular response to enteric LPS in cells expressing human receptor proteins in a dose-dependent manner (Figure 4.4). The effect of RsLPS on NF-κB activation was, therefore, consistent with its previously determined effect on TNFα production by human or equine monocytic cells.23

The currently accepted model of LPS interaction with cellular receptors comprises at least 4 proteins: Lipopolysaccharide binding protein (LBP), CD14, TLR4 and MD-2. LBP is important for transport of LPS monomers to the cellular surface,26 but may not be part of the ligand-receptor complex involved in signal transduction. CD14 is responsible for LPS binding, and antibodies to CD14 can inhibit LPS binding and signal transduction.27, 28 In the absence of membrane-expressed CD14, soluble CD14 contained in plasma can serve to confer responsiveness to LPS to cells.29 Because we could show that FBS eliminated the need for transfection with CD14, we limited the serum concentration in our assays. The TLR4/MD-2 complex represents the receptor portion that initiates signal transduction.9, 30 TLR4 is the only transmembrane protein in the LPS receptor complex, and is thought to associate with MD-2, a secreted protein, via its extracellular portion.31

A surprising finding in the study reported here was that RsLPS stimulated NF-κB activation via equine receptors independent of CD14, while the presence of all three proteins – CD14, TLR4 and MD-2 – was required to obtain a response to E. coli LPS (Figure 4.3). This
finding suggests that the interaction of RsLPS with LPS receptor proteins may differ from that of enteric LPS. For example, one might speculate that RsLPS forms complexes with LBP but is transferred directly to TLR4/MD-2 without requiring interaction with CD14. Alternatively, a protein other than CD14 could be responsible for binding of RsLPS to the cell surface. Because HEK293 cells that expressed only the reporter enzymes did not respond to RsLPS, it is unlikely that RsLPS effected NF-κB activation by interaction with receptors other than TLR4 and MD-2. A caveat concerning our results is that we did not eliminate FBS from our experiments completely, and that, therefore, the effect of RsLPS on cells expressing equine TLR4/MD-2 but not CD14 could have been mediated by small amounts of sCD14 in our assays. We reduced the concentration of FBS in our assays arbitrarily to 0.025% in an attempt to limit the effect of sCD14 while still supplying LBP. Our finding that, given these conditions, HEK293 cells did not respond to E. coli LPS unless transfected with CD14, TLR4 and MD-2, suggested that our approach was successful in eliminating the effect of sCD14 contained in FBS. However, it is possible that lower concentrations of sCD14 are sufficient to effect a response to RsLPS than to LPS from E. coli. Purified or recombinant equine LBP and sCD14 could be used to resolve this issue, however, neither protein is available at this time. An alternative approach would be to repeat our experiments in the presence of an antibody directed against equine CD14. Again, antibodies against equine CD14 are not available to date, and we have not been able to identify antibodies against CD14 from other species that block the response of equine cells to LPS (unpublished observation).

By combining human and equine receptor proteins, we determined that both TLR4 and MD-2 need to be of equine origin for the cells to respond to RsLPS (Figure 4.6). Not surprisingly, considering the apparent lack of contribution of CD14 to the cellular response, the
species origin of CD14 was inconsequential (Figure 4.5). The lack of activation by RsLPS of cells expressing chimeric receptor complexes was unlikely due to expression of dysfunctional receptor complexes, because all protein combinations yielded receptor complexes that conferred responsiveness to *E. coli* LPS (Figure 4.5 and 4.6). Several studies have previously addressed species-specific responses to different LPS or lipid A compounds, and, in agreement with our findings, CD14 was not responsible for species-specific responses to lipid IVa and RsLA in transfected cells. The significance of TLR4 and/or MD-2 for species-specific responses to LPS remains, however, controversial. Murine TLR4 restored the response of cells from C3H/HeJ mice lacking functional endogenous TLR4 to lipid IVa, and over-expression of human TLR4 in Chinese hamster ovary cells expressing human CD14 abolished the cells’ response to lipid IVa and RsLA. Further, over-expression of hamster TLR4 in human THP-1 cells rendered the cells responsive to lipid IVa and RsLA. In the latter study, 100-fold higher concentrations of lipid IVa and RsLA as compared to enteric LPS were required to stimulate a response. It remains speculative whether higher concentrations of RsLPS in our study would have stimulated cells expressing eTLR4/hMD-2, or hTLR4/eMD-2. In contrast to these reports suggesting that TLR4 determines species-specific responsiveness to LPS, expression of human MD-2 in combination with murine TLR4 in BaF3 cells changed the biological activity of lipid IVa from an agonist to an antagonist. Similarly, MD-2 and not TLR4 was responsible for the murine response to lipid IVa in transfected HEK293 cells. Our findings differ from these reports but are in agreement with a report that both TLR4 and MD-2 contribute to the species-specific response to specific *Salmonella* lipid A structures.

In conclusion, we show here that expression of equine receptor proteins in HEK293 cells can be used to evaluate NF-κB activation in response to LPS stimulation, and that equine
receptor proteins are able to form functional LPS receptor complexes with human proteins. Our experiments further suggest that TNFα production by equine cells in response to RsLPS is stimulated at the level of NF-κB. Surprisingly, we determined that stimulation of transfected cells by RsLPS occurred independent of CD14, a finding that warrants further investigation. Finally, we show that TLR4 and MD-2 are not able to elicit the equine-specific response to RsLPS individually, but that both proteins need to be of equine origin in order to recognize RsLPS as an agonist.

REFERENCES


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**Figure 4.1:** RT-PCR of RNA from transfected and non-transfected HEK293 cells to demonstrate transcription of receptor plasmid sequences into mRNA. HEK293 cells were transfected with human (HEK-HR) or equine (HEK-ER) receptor plasmids (CD14, TLR4, MD-2) and RNA isolated as described in materials and methods. RNA was subjected to RT-PCR using primers designed to amplify human (h) or equine (e) plasmid receptor sequences. Primer sequences are described in the materials and methods section. Non-transfected cells (HEK-NT) served as negative controls. MW = molecular weight marker, bp = base pairs, RT-PCR = reverse transcription and polymerase chain reaction.
**Figure 4.2:** LPS from *Rhodobacter sphaeroides* (RsLPS) stimulates NF-κB activation in HEK293 cells expressing equine receptor proteins, but not in cells expressing human proteins. HEK293 cells were transfected with plasmids encoding CD14, TLR4 and MD-2 of equine or human origin, respectively, and luciferase reporter plasmids. Control cells were transfected only with reporter plasmids. Cells were stimulated for 4 hours with LPS from *E. coli* O55:B5 or RsLPS and relative light units (RLU) determined as the ratio of firefly/renilla luciferase activity. Results are shown as the fold-increase (FI) of RLU over baseline, which was determined by incubation of cells with media alone. Results are the mean ± standard deviation of at least 4 experiments, except for the response to RsLPS at a concentration of 100 ng/ml in cells expressing equine proteins and in control cells, which represents 2 experiments. * indicate significant increases of RLU over baseline.
Figure 4.3: CD14 is not required for stimulation of NF-$\kappa$B activation by *Rhodobacter sphaeroides* LPS (RsLPS) in HEK293 cells expressing equine receptor proteins. HEK293 cells were transfected with individual plasmids or plasmid combinations encoding CD14, TLR4 or MD-2 of equine origin, and with luciferase reporter plasmids. Cells were stimulated for 4 hours with LPS from *E. coli* O55:B5 (10 ng/ml) or RsLPS (10 ng/ml) and relative light units (RLU) were determined as a measure of NF-$\kappa$B activation. RLU were determined as the ratio of firefly/renilla luciferase activity. Results are shown as the fold-increase of RLU over baseline, which was determined by incubation of cells with media alone. Results are the mean ± standard deviation of 3 experiments using RsLPS and 5 experiments using *E. coli* LPS. * indicate significant increases of RLU over baseline.
Figure 4.4: LPS from *Rhodobacter sphaeroides* (RsLPS) inhibits NF-κB activation in response to *E. coli* LPS in HEK293 cells expressing human receptor proteins. HEK293 cells were transfected with plasmids encoding human CD14, TLR4 and MD-2, and with luciferase reporter plasmids. Cells were incubated for one hour with RsLPS at the indicated concentrations, then *E. coli* LPS (10 ng/ml) was added and cells were incubated for an additional 4 hours. Relative light units (RLU) were determined as the ratio of firefly/renilla luciferase activity. Results are presented as the relative fold-increase (FI) of RLU over baseline, which was determined by incubation of cells with media alone. 100% represents the FI of RLU over baseline in response to *E. coli* LPS alone. * indicate significant differences from *E. coli*-stimulated cells, □ indicate significant differences from baseline. Results represent averages and standard deviations of 3 experiments.
Figure 4.5: CD14 is not responsible for equine-specific NF-κB activation in response to *Rhodobacter sphaeroides* LPS (RsLPS). HEK293 cells were transfected with combinations of CD14, TLR4 and MD-2 of equine or human origin, and luciferase reporter plasmids. Equine plasmids are underlined to facilitate differentiation from human plasmids. Cells were stimulated with LPS from *E. coli* (10 ng/ml) or RsLPS (10 ng/ml or 100 ng/ml) for 4 hours, and relative light units (RLU) determined as the ratio of firefly/renilla luciferase activity. Results are shown as the fold-increase (FI) of RLU over baseline, which was determined by incubation of cells with media alone. * indicate significant increases of RLU over baseline. Results are the mean ± standard deviation of 3 experiments using *E. coli* LPS and RsLPS at a concentration of 10 ng/ml, and of 2 experiments using RsLPS at a concentration of 100 ng/ml.
Figure 4.6: TLR4 and MD-2 need to be of equine origin to render transfected HEK293 cells responsive to *Rhodobacter sphaeroides* LPS (RsLPS). HEK293 cells were transfected with combinations of CD14, TLR4 and MD-2 of equine or human origin, as indicated, and luciferase reporter plasmids. Equine plasmids are underlined to facilitate differentiation from human plasmids. Cells were stimulated with LPS from *E. coli* (10 ng/ml) or RsLPS (10 ng/ml or 100 ng/ml) for 4 hours, and relative light units (RLU) determined as the ratio of firefly/renilla luciferase activity. Results are shown as the fold-increase (FI) of RLU over baseline, which was determined by incubation of cells with media alone. * indicate significant increases of RLU over baseline. Results are the mean ± standard deviation of 3 experiments using *E. coli* LPS and RsLPS at a concentration of 10 ng/ml, and of 2 experiments using RsLPS at a concentration of 100 ng/ml.
CHAPTER 5
LIPOPOLYSACCHARIDES FROM RZIZOBIUM GALEGAE AND RZIZOBIUM SIN-1 ARE AGONISTS IN EQUINE CELLS AND ACT VIA THE LIPOPOLYSACCHARIDE RECEPTOR COMPLEX

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ABSTRACT

Endotoxemia is a common and serious complication of equine diseases, and investigation of novel treatment strategies and research tools is of interest. Lipopolysaccharide (LPS) antagonists are molecules of LPS structure that do not activate inflammatory cells but instead inhibit the cellular response to endotoxin. Species-specific differences in the response to certain LPS antagonists have been recognized. Here, the biological activities of two rhizobial LPS compounds (LPS from *Rhizobium galegae* and *Rhizobium Sin-1*) were investigated by determining their ability to induce tumor necrosis factor α (TNFα) production in equine monocyte preparations and in a human monocytic cell line, Mono Mac 6. We further performed binding assays to investigate competition for binding among LPS compounds, and transfection experiments to investigate the effect of rhizobial compounds on nuclear factor κB (NF-κB) activation. Rhizobial LPS inhibited endotoxin-induced TNFα production in human cells but stimulated TNFα production in equine cells. Rhizobial LPS competed with enteric LPS for binding to equine monocytes. In HEK293 cells expressing either human or equine LPS receptor proteins, rhizobial LPS stimulated NF-κB activation and only partially inhibited NF-κB activation in response to enteric LPS. We conclude that LPS from *Rhizobium galegae* and *Rhizobium Sin-1* are LPS antagonists in human cells but are low-potency agonists of at least moderate efficacy in equine cells. Rhizobial LPS exert their stimulatory effects in equine cells via the LPS receptor complex consisting of CD14, TLR4 and MD-2. The antagonistic effect of rhizobial LPS in human cells may not be due to a competitive mechanism.
INTRODUCTION

Endotoxemia is an important and life-threatening complication of many equine diseases, most notably neonatal sepsis caused by gram-negative bacteria\(^1\) and acute gastrointestinal disease, or colic, associated with compromised intestinal barrier function.\(^2,3\) Horses are exquisitely sensitive to endotoxin, and understanding of the mechanisms responsible for cellular activation by endotoxin is a vital step in the development of new methods of treatment.

Endotoxin is an integral outer membrane component of the Gram-negative bacterial cell wall. Endotoxin has a lipopolysaccharide (LPS) structure and is structurally divided into an O-chain polysaccharide, a core oligosaccharide and the lipid A portion, which represents the active moiety.\(^4,5\) Based on the presence or absence of an O-chain polysaccharide, smooth LPS containing the O-chain and rough mutants without an O-polysaccharide are differentiated. The lipid A structure of enteric LPS is well conserved among bacterial species and is characterized by a disaccharide (diglucosamine) backbone in $\beta(1\rightarrow6)$ linkage, that carries negatively charged phosphate groups at the 1- and 4’-ends and is asymmetrically substituted with fatty acyl chains.\(^6\) All structural elements of lipid A (i.e. the negatively charged backbone and the number, type and possibly distribution of fatty acyl chains) are operational in determining endotoxic activity.\(^7\)

LPS stimulates inflammatory cells such as monocytes or macrophages by interacting with a cellular receptor complex consisting of at least 3 proteins. CD14 (cluster differentiation antigen 14) is instrumental in LPS binding, while TLR4 (Toll-like receptor 4) in combination with MD-2 represents the signal transducing portion of the receptor complex.\(^8-10\) In addition to these receptor proteins, LPS-mediated cellular stimulation requires the presence of a serum protein, lipopolysaccharide binding protein or LBP, which transports LPS monomers to the cellular surface and therefore greatly enhances the potency of LPS.\(^11\) LPS (or lipid A) antagonists are
molecules of LPS (or lipid A) structure that inhibit the response of inflammatory cells to endotoxin without themselves stimulating an agonistic response. LPS antagonists purportedly exert these effects by competing with endotoxin for binding to transport proteins and the cellular LPS receptor complex; however, additional mechanisms have also been proposed.

Rhizobia are nitrogen-fixing Gram-negative bacteria, which form symbiotic relationships with plants in the form of root nodules. Rhizobia produce LPS whose lipid A portion is structurally very distinct from enteric lipid A. Characteristic features of rhizobial lipid A include atypical backbone monosaccharides, lack of backbone phosphorylation and the presence of a long (28 carbon) fatty acyl chain. Because of their unique structural characteristics, rhizobial LPS and/or lipid A compounds are an attractive tool for investigating LPS structure-function relationships. In the study reported here, we investigated the biological effects of LPS from two rhizobial species, Rhizobium spp. Sin-1 (R. Sin-1) and Rhizobium galegae (R. galegae). Analysis of lipid A preparations from R. Sin-1 yields a mixture of structures, which differ with regards to the disaccharide backbone and the acylation pattern. The proximal backbone residue is either a 2-aminoglucono-1,5-lactonosyl residue or a 2-amino-2,3-dideoxy-D-erythro-hex-2-enono-1,5-lactone residue, while the distal residue is always glucosamine. Consistent with the lipid A structures described for other Rhizobia, the backbone of R. Sin-1 is not phosphorylated. Different fatty acyl substitution patterns can be discerned, which do not appear with the same frequency. The major fraction of molecules, independent of the backbone structure, carries the following fatty acyl substituents: position 2’ is acylated with β-hydroxypalmitoyl (3-OH-C16:0) that is O-acylated by a secondary 27-hydroxyoctacosanoid acid (27-OH-C28:0); position 2 carries predominantly 3-OH-C16:0; and position 3’ is either acylated with β-hydroxymyristoyl (3-OH-C14:0) or does not carry an acyl chain. A small fraction of lipid A molecules is
substituted with 3-OH-C14:0 in position 3 and/or β-hydroxystearoyl (3-OH-C18:0) or β-hydroxyoctadecenooyl (3-OH-C18:1) in positions 2 and 2'.\textsuperscript{20} The structure of lipid A from \textit{R. galegae} has not yet been published, however, it is very similar to that of lipid A from \textit{R. Sin-1}. (R. Carlson, personal communication)

In the study reported here, we determined that LPS from \textit{R. Sin-1} and \textit{R. galegae} induce NF-κB activation and TNFα production in equine cells, while both inhibit TNFα production by human cells in response to enteric LPS. We further show that in individual horses, LPS from \textit{R. galegae} can be classified as a partial agonist and partially inhibits monocyte TNFα production in response to enteric LPS. Using radioligand binding studies and transfection experiments, we further provide evidence supporting the involvement of the LPS receptor complex, consisting of CD14, TLR4 and MD-2, in the cellular responses to rhizobial LPS.

**MATERIALS AND METHODS**

**Lipopolysaccharides:** LPS from \textit{E. coli} O55:B5 and [\textsuperscript{3}H]LPS from \textit{E. coli} K12 strain LCD25 were purchased from List Biologicals, Campbell, CA. LPS from \textit{R. Sin-1} and \textit{R. galegae} were provided by Dr. R. Carlson, Athens, GA.

**Calculation of lipid A equivalent concentration:** Because dilutions of LPS were made based on the absolute weight of LPS rather than its molecular weight, we calculated the concentration of lipid A equivalents per mg LPS based on the amount of β-hydroxymyristic acid present in \textit{E. coli} LPS and the amount of β-hydroxypalmitic acid present in LPS from \textit{R. galegae} and \textit{R. Sin-1}. According to analyses performed in Dr. R. Carlson’s laboratory at the University of Georgia (Athens, GA), LPS from \textit{E. coli} O55:B5 contained 17 µg lipid A equivalents (equiv)/mg, LPS
from *R. galegae* contained 31 µg lipid A equiv/mg, and LPS from *R. Sin-1* contained 41 µg lipid A equiv/mg.

**Cells:** Equine mononuclear cells were isolated using density gradient centrifugation as described previously. Briefly, blood was collected into ethylene diamine tetraacetic acid (EDTA)-coated blood collection bags or 50 ml conical tubes containing EDTA (2.5 nmol EDTA/ml blood). Erythrocytes were allowed to sediment by gravity, and the leukocyte-rich plasma was removed and centrifuged (1200 rpm, 5 min). After the supernatant was decanted, the leukocyte pellet was suspended in phosphate-buffered saline (PBS) and layered over a mixture of Percoll (54%)(Sigma-Aldrich, St Louis, MO), 1.5 M NaCl (6%) and RPMI-1640 (40%). The tubes were centrifuged (1800 rpm, 15 min) and the mononuclear cell band removed by careful aspiration. Cells were washed twice in PBS and suspended in RPMI-1640 containing penicillin/streptomycin and 5% fetal bovine serum (FBS). Mono Mac 6 cells were provided by Dr. H.W.L. Ziegler-Heitbrock (University of Munich, Germany). Cells were grown in RPMI-1640 medium with 10% FBS, penicillin/streptomycin and OPI media supplement containing oxaloacetate, pyruvate and bovine insulin. For 48 hours prior to experiments, cells were incubated with 1α,25-dihydroxy-vitamin D₃ (0.01 µg/ml cells) to enhance expression of CD14.

**Cell stimulation:** Equine mononuclear cells were plated at a density of 3 x 10⁶ cells/well in 12-well polystyrene plates and monocytes isolated by cytoadherence for 2 hours. Non-adherent cells were removed by washing 3 times in PBS, and adherent monocytes cultured in RPMI-1640 containing penicillin/streptomycin and 5% FBS. In previous studies performed in our laboratory, > 85% of adherent cells isolated in this fashion have been identified as monocytes based on non-specific esterase staining. Mono Mac 6 cells were pelleted by centrifugation (1400 rpm, 5 min), washed 3 times in PBS and then suspended in complete culture medium as described above.
Aliquots of 0.5 x 10^6 cells/tube were added to 12 x 75 mm polypropylene tubes for stimulation with LPS. LPS stock solution (1 mg/ml in PBS) was sonicated for 60 sec and diluted in tissue culture medium before use. Cells were incubated with LPS for 6 hours at 37°C, 5% CO₂. To determine the effect of serum on LPS-induced production of TNFα, cells were washed once in FBS-free medium and then stimulated with LPS in the absence of FBS. To investigate the effect of rhizobial LPS compounds on cellular stimulation by enteric LPS, cells were incubated with rhizobial LPS for one hour prior to addition of *E. coli* LPS, and then incubated for an additional 6 hours. Supernatants were collected for TNFα assay; Mono Mac 6 cells were pelleted prior to collection of cell supernatants.

**TNFα assay and data analysis:** TNFα activity in equine monocyte cell supernatants was determined by modification of an *in vitro* cytotoxicity bioassay using the murine fibrosarcoma cell line WEHI-164 clone 13 as described previously.²⁴ TNFα production by Mono Mac 6 cells was measured using a biotinylated enzyme-linked immunosorbent assay (ELISA) as described previously.²⁵ Because of the differences in the assay types, values of equine TNFα are reported as activities in [units/ml], while those for human TNFα are reported as concentrations in [pg/ml]. Dose-response curves were fit using GraphPad Prism software (GraphPad Prism version 2.0, GraphPad Software, Inc., San Diego, CA). Maximal TNFα response and EC₅₀ (i.e. the concentration of LPS eliciting a half-maximal response) were compared between LPS compounds using non-parametric analysis with the Kruskal-Wallis test. Pairwise comparisons were performed using the Wilcoxon rank sum test. For antagonism experiments, IC₅₀ values (i.e. the rhizobial LPS concentration inhibiting TNFα production by 50%) were compared using the Wilcoxon rank sum test.
**Radioligand binding assay and data analysis:** Mono Mac 6 cells were washed once in ice-cold HNE buffer (20 mM HEPES pH 7.4, 150 mM NaCl, 1 mM EDTA) and suspended in SEBDEF buffer (20 mM HEPES pH 7.4, 150 mM NaCl, 1 mM EDTA, 10 mM NaN₃, 2 mM NaF, 5 mM deoxyglucose, 300 µg/ml bovine serum albumin). Equine monocytes were isolated in Petri dishes by cytoadherence for 2 hours, and non-adherent cells removed by washing 3 times in ice-cold HNE buffer. Adherent cells were harvested by careful scraping with a rubber policeman, washed once in ice-cold HNE buffer, and suspended in SEBDEF buffer. Cells were incubated in SEBDEF buffer for 30 min at 37°C, 5% CO₂ to deplete adenosine triphosphate (ATP) and prevent receptor and ligand internalization during experiments. At the end of the incubation, cells were centrifuged and suspended in RPMI-B (RPMI 1640 containing 10 mM NaN₃, 2 mM NaF, 5 mM deoxyglucose, 300 µg/ml bovine serum albumin), and aliquots of 10⁶ cells were added to microcentrifuge tubes. Total assay volume was 0.5 ml. [³H]LPS (stock solution 0.1 mg/ml in 0.1% triethylamine/10 mM Tris-HCl) was sonicated for 60 sec and diluted in RPMI-B immediately before use. [³H]LPS was incubated with unlabeled competitor and FBS (final concentration in assay 15%) for 30 min at room temperature before being added to the cells. Cells were incubated with LPS for 60 min at 37°C on an elliptical shaker. Reactions were stopped by addition of ice-cold HNE buffer (750 µl) followed immediately by centrifugation (15,000 rpm, 2 min). Supernatants were removed and cell pellets washed once in ice-cold HNE buffer before being lysed in scintillation fluid (ScintiVerse®, Fisher Scientific, Fair Lawn, NJ). Radioactive counts of cell-bound [³H]LPS were determined by liquid scintillation counting using an LS 6500 scintillation counter (Beckman Coulter Inc.). Cell-associated counts were plotted against the concentration of unlabeled competitor and curves fit using GraphPad Prism software. Values for IC₅₀ (i.e. the concentration of competitor that reduced binding of radioligand by 50%)
were determined from the curves and compared between compounds using 95% confidence intervals.

**Plasmids:** Human CD14 in pcDNA 3.1, human MD-2 in pEF-BOS and the firefly luciferase reporter plasmid pELAM were a generous gift from D. Golenbock, Worcester, MA. Human TLR4 in pEF-BOS was a generous gift from K. Miyake, Tokyo, Japan. The renilla luciferase reporter plasmid pTKRL was purchased from Promega Corp., Madison, WI. Equine CD14 was cloned into pcDNA 3.1 (Invitrogen, Carlsbad, CA) in our laboratory. Equine TLR4 and equine MD-2 were cloned into pBluescript (Stratagene, La Jolla, CA) in our laboratory. For subcloning of equine TLR4 and equine MD-2 into pEF-BOS, insert clones were produced by PCR using the Advantage™-HF2 PCR kit (BD Biosciences Clontech, Palo Alto, CA). PCR primers were used that introduced *XhoI* and *BamHI* restriction sites at the 5' and 3' ends of the inserts, respectively. For TLR4, the forward and reverse primer sequences were (5'→3') 5'-
gatctcaggttctgggacagaaatgc-3' and (3'→5') 5'-gatggatccggtagagtttctgcatcg-3'. For MD-2, the primer sequences were (5'→3') 5'-gatctcaggttaaatgtagttggtgta-3' and (3'→5') 5'-
gatggatccattgaaactaggttgta-3'. Inserts were digested with *XhoI* and *BamHI* (Promega Corp., Madison, WI), submitted to gel electrophoresis and purified from the gel using the QIAquick® gel extraction kit (Qiagen, Valencia, CA). pEF-BOS plasmid containing human TLR4 was digested with *XhoI* and *BamHI*, and vector separated from insert by gel electrophoresis and purification. New inserts were ligated into the vector using the TaKaRa DNA ligation kit, ver.2 (TAK6022, Takara Mirus Bio, Kyoto, Japan and Madison, WI). Ligation reactions were purified using the QIAquick® PCR purification kit (Qiagen, Valencia, CA), and Top10 competent *E. coli* (Invitrogen, Carlsbad, CA) were transformed using electroporation. Plasmid preparation was performed using the QIAprep® miniprep kit and EndoFree® plasmid maxi kit (Qiagen, Valencia, CA).
Correctly ligated plasmids were confirmed using restriction digestion and sequencing techniques.

**RNA isolation and RT-PCR:** HEK293 cells were obtained from American Type Culture Collection (ATCC), Manassas, VA. HEK 293 cells were maintained in Minimum Essential Medium (MEM) containing penicillin/streptomycin and 10% FBS. Cells were grown in T25 culture flasks to approximately 50-60% confluency and were transfected with equine or human receptor protein plasmids. Cells were transfected using 0.5 µg each of CD14, TLR4 and MD-2 and PolyFect® reagent (Qiagen, Valencia, CA) at a ratio of 15 µl PolyFect®/µg plasmid DNA. Fresh tissue culture medium was applied 6 hours after transfection, and cells were maintained for approximately 36 hours before RNA isolation. RNA was isolated using the Absolutely RNA™ kit (Stratagene, La Jolla, CA). RNA was submitted to restriction digestion using AluI (Promega Corp., Madison, WI) to destroy remaining plasmid DNA. RNA was used as a template for reverse transcription and polymerase chain reaction (RT-PCR) using the QIAGEN® One-Step RT-PCR kit (Qiagen, Valencia, CA). Primers used to amplify fragments were: for human CD14: (5’→3’) 5’-aagccttccagtgtgtgtgc-3’ and (3’→5’) 5’-ttcgcccagtgattgc-3’; for human TLR4: (5’→3’) 5’-tcattttccctggtgagtgtgac-3’ and (3’→5’) 5’-ggacaagccattgaagatgcc-3’; for human MD-2: (5’→3’) 5’-gaagctcagaagcattggtgc-3’ and (3’→5’) 5’-ggtgtaggtgacaactccag-3’; for equine CD14: (5’→3’) 5’-gtcactcactgccttttcc-3’ and (3’→5’) 5’-tcagggtcagatgatgattcc-3’; for equine TLR4: (5’→3’) 5’-tcaatggtggttggtctggag-3’ and (3’→5’) 5’-cctgtttcaagtggaatgctgg-3’; for equine MD-2: (5’→3’) 5’-caatgcgtaaagaggttatttgcc-3’ and (3’→5’) 5’-gggtgtgtgtgatatgcaatg-3’. Control PCR reactions not including a reverse transcription step were performed with the same primers and templates using the MasterTaq kit (Eppendorf, Hamburg, Germany). RNA isolated from human Mono Mac 6 cells and equine monocytes served as positive control.
templates, and RNA isolated from HEK293 cells served as a negative control template. For detection, PCR products were submitted to gel electrophoresis on a 1.5% agarose gel.

**HEK transfection and stimulation and data analysis:** HEK293 cells were seeded in 96-well polystyrene tissue culture plates at approximately 20,000 cells/well to achieve a density of 60-80% at the time of transfection. The following day, cells were transfected with receptor plasmids (1 ng each/well) using PolyFect® reagent (Qiagen, Valencia, CA) at a ratio of 1.5 µl PolyFect®/100 ng plasmid DNA. Cells also were transfected with two luciferase reporter plasmids: pELAM, encoding an NF-κB inducible firefly luciferase (50 ng/well), and pTKRL, encoding a constitutively active renilla luciferase (5 ng/well). Total DNA in the transfections was adjusted to 100 ng/well using empty pcDNA3.1 plasmid. Following transfection for 6 hours, fresh tissue culture medium was added and cells were cultured for 36-42 hours prior to stimulation with LPS. Recombinant human TNFα (10 ng/ml) was used as a control stimulus to assess luciferase reporter function. LPS was diluted in tissue culture medium containing 0.5% FBS and incubated for 15-30 min at room temperature. LPS was then diluted 20-fold in serum free medium and added to cells that had been washed once in serum-free medium. The final concentration of FBS in the assays was therefore 0.025%. Cells were incubated with LPS for 4 hours, then washed three times in PBS and lysed in passive lysis buffer (50µl/well) provided with the luciferase reporter assay system kit. 20µl of cell lysate/well was transferred to a 96-well plate suitable for luminescence reading and luciferase activity determined using a dual-luciferase® reporter assay system (Promega Corp., Madison, WI) and a Fluoroskan Ascent FL plate luminometer (Thermo Labsystems). Individual experiments were performed in duplicate or triplicate with treatments assigned randomly to the wells in a 96-well plate, the number of repetitions (n) for each experiment is indicated in the results section.
**Data analysis:** For each sample, the firefly luciferase value was divided by the renilla luciferase value and the ratio reported in relative light units (RLU). Baseline RLU were determined from measurements of luciferase activity in unstimulated cells. Data were analyzed using analysis of variance in a randomized block design to account for an effect of experiment repetitions on different days. RLU of LPS-stimulated cells were compared to those of unstimulated cells using the least square means. Comparisons were only performed within groups of cells expressing the same receptor proteins, as baseline values differed depending on the transfection status of cells. Differences were considered significant at $p \leq 0.05$. In the graphs, fold-increases of RLU over baseline ($F_{BL}$) are reported, which were calculated by dividing RLU of LPS-stimulated cells by baseline RLU.

**RESULTS**

**LPS from *R. galegae* and *R. Sin-1* inhibit** **TNF$\alpha$ production by MonoMac 6 cells stimulated with *E. coli* LPS**

Mono Mac 6 cells responded strongly to *E. coli* LPS, while no TNF$\alpha$ was produced in response to LPS from *R. galegae* and *R. Sin-1* up to concentrations of 10 $\mu$g/ml. Maximal TNF$\alpha$ concentration in cell media after stimulation with *E. coli* LPS reached an average ($\pm$ standard deviation) of 1452 $\pm$ 644 pg/ml with a median of 1463 pg/ml and a range of 664 to 2219 pg/ml ($n=4$). Average $EC_{50}$ was 0.51 $\pm$ 0.19 ng/ml, median $EC_{50}$ was 0.46 ng/ml and the range was 0.35 to 0.77 ng/ml. In the absence of serum, maximal TNF$\alpha$ production was significantly reduced while $EC_{50}$ did not differ significantly (Table 5.1). The difference in maximal TNF$\alpha$ production and $EC_{50}$ between cells cultured in the presence of 5% FBS and 10% FBS, respectively, was not
significant (maximal TNF$\alpha$ concentration 715.5 pg/ml and 663.6 pg/ml; EC$_{50}$ 0.423 ng/ml and 0.352 ng/ml, n=1).

LPS from *R. galegae* and *R. Sin-1* completely inhibited production of TNF$\alpha$ by cells incubated with 10 ng/ml *E. coli* LPS (Figure 5.1); IC$_{50}$ for *R. galegae* and *R. Sin-1* LPS did not differ significantly. Average IC$_{50}$ for *R. galegae* LPS in 5 experiments was 479.48 ± 440.8 ng/ml (14.86 ± 13.67 ng lipid A equiv/ml), median IC$_{50}$ was 339.5 ng/ml (10.53 ng lipid A equiv/ml) and the range was 50.31 to 1202 ng/ml (1.56 to 37.26 ng lipid A equiv/ml). For *R. Sin-1* LPS, average IC$_{50}$ in 8 experiments was 388.88 ± 527.68 ng/ml (15.94 ± 21.64 ng lipid A equiv/ml), median IC$_{50}$ was 195.45 ng/ml (8.01 ng lipid A equiv/ml) and the range was 68.95 to 1665 ng/ml (2.83 to 68.27 ng lipid A equiv/ml).

**LPS from *R. galegae* and *R. Sin-1* stimulate TNF$\alpha$ production by equine monocytes**

Considerable variation occurred among horses with regards to the maximal TNF$\alpha$ production by monocytes in response to LPS. Representative dose response curves for *E. coli* LPS and rhizobial LPS from one experiment are presented in Figure 5.2; the combined data for all experiments are shown in Table 5.2. The effect of serum deprivation on cellular stimulation by *E. coli* LPS was similar to that observed in Mono Mac 6 cells, the results are shown in Table 5.3.

Dose-response curves for rhizobial LPS compounds did not permit determination of maximal TNF$\alpha$ activities in all cases, because an exponential increase in TNF$\alpha$ production occurred at high concentrations of LPS in several experiments. In those experiments, EC$_{50}$ values derived from artificially constrained curves could only be considered as estimates, because EC$_{50}$ changed depending on the value used to constrain the maximum of the curve. For 10 of 11
experiments with *R. galegae* LPS, the data could be fit properly and only parameters derived from these curves are included in Table 5.2. In contrast, proper curve fitting was possible in only 5 of 9 experiments with *R. Sin-1* LPS; again, only these data are included in Table 5.2. Using only data derived from non-constrained curves for comparison, EC$_{50}$ for both *R. galegae* and *R. sin-1* LPS were significantly greater than EC$_{50}$ for *E. coli* LPS, but did not differ significantly from each other. Due to the considerable variation in TNF$\alpha$ production among horses, maximal TNF$\alpha$ activity responses as an indicator of efficacy of the different LPS compounds were only compared within individual experiments, where compounds were evaluated side by side with monocytes from the same horse. *E. coli* LPS and *R. galegae* LPS were compared in 7 horses, with the curves allowing proper fitting for both compounds in 5 horses. Using comparison of 95% confidence intervals, maximal TNF$\alpha$ activity did not differ significantly between the compounds in two horses, was higher for *R. galegae* LPS than *E. coli* LPS in two horses, and was higher for *E. coli* LPS in one horse. Similarly, *E. coli* LPS and *R. Sin-1* LPS were compared in 7 horses, but only two experiments yielded properly fit curves for both compounds. Maximal TNF$\alpha$ activity did not differ significantly between *E. coli* LPS and *R. Sin-1* LPS in these horses. Responses to *R. galegae* LPS and *R. Sin-1* LPS were compared in 5 horses, and two experiments yielded properly fitted curves for both compounds. Maximal TNF$\alpha$ activity did not differ significantly between the compounds in these experiments.

Because comparisons of maximal TNF$\alpha$ production suggested that *R. galegae* LPS may be a partial agonist in some horses (i.e., it induced lower maximal production of TNF$\alpha$ than *E. coli* LPS), monocytes from 11 horses were stimulated with 1 ng/ml of *E. coli* LPS in the presence of increasing concentrations of *R. galegae* LPS. Unstimulated cells, cells stimulated with 1 ng/ml *E. coli* LPS alone, and cells stimulated with *R. galegae* LPS alone served as
controls. In monocytes from 5 horses, TNFα production in response to *E. coli* LPS was not reduced by *R. galegae* LPS; rather, addition of *R. galegae* LPS tended to increase TNFα production above that measured in response to *E. coli* LPS alone. In cell preparations from 4 of these 5 horses, *R. galegae* LPS by itself induced maximal production of TNFα that equaled or exceeded that in response to 1 ng/ml *E. coli* LPS. In contrast, *E. coli* LPS-induced TNFα production was reduced by the presence of *R. galegae* LPS in monocytes from the other 6 horses. In 2 experiments, TNFα production was reduced to a level equal to the maximal TNFα activity induced by *R. galegae* LPS alone, which was consistent with the designation of *R. galegae* LPS as a partial agonist (Figure 5.3). In one horse, *R. galegae* LPS reduced TNFα production in response to *E. coli* LPS although maximal TNFα production in response to *R. galegae* LPS alone exceeded that in response to 1 ng/ml *E. coli* LPS. In the other horses, maximal TNFα production in response to *R. galegae* LPS could not be defined definitively. In combination, these experiments show that LPS from *R. galegae* and *R. Sin-1* induce TNFα production in equine monocytes, and that the efficacy of LPS from *R. galegae* differs among horses.

**LPS from *R. galegae* and *R. Sin-1* compete for binding of *E. coli* LPS to equine monocytes**

To investigate whether the rhizobial LPS compounds compete with enteric LPS for binding to equine monocytes, equilibrium competition binding experiments were performed, in which equine monocytes were incubated with radioligand LPS ([3H]LPS from *E. coli* K12 LCD25) in the presence of increasing concentrations of unlabeled rhizobial LPS. Unlabeled LPS from *E. coli* O55:B5 was used as a control competitor that would be expected to bind to the same site as the radioligand. Data from a representative experiment are presented in Figure 5.4, and IC50 values obtained in 3 experiments are presented in Table 5.4. The 95% confidence intervals
for the IC$_{50}$ values were compared between compounds to identify significant differences. In 2 of the 3 experiments, $E. \text{coli}$ LPS was a significantly more potent inhibitor of binding than either $R. \text{galegae}$ LPS and $R. \text{Sin-1}$ LPS, whose IC$_{50}$ values did not differ significantly. In one experiment, IC$_{50}$ for $E. \text{coli}$ LPS differed significantly from that for $R. \text{galegae}$ LPS, but not from that for $R. \text{Sin-1}$ LPS, which was likely attributable to a large confidence interval for the IC$_{50}$ of $R. \text{Sin-1}$ LPS. Overall, the results of these experiments indicate that all 3 LPS compounds compete with radiolabeled LPS for binding to equine monocytes, and that the affinity for binding of $E. \text{coli}$ LPS is higher than for the rhizobial compounds.

**Rhizobial LPS compounds stimulate NF-κB activation in HEK 293 cells transfected with human or equine LPS receptor proteins**

To investigate whether rhizobial LPS compounds stimulate NF-κB activation, a dual luciferase reporter assay system was used in transfected HEK293 cells. HEK293 cells were transfected with plasmids encoding equine or human LPS receptor proteins (CD14, TLR4, MD-2) and luciferase reporter plasmids, and were stimulated with LPS. Cells transfected with reporter plasmids alone were used as controls to determine receptor-independent cellular stimulation by the LPS compounds. The results are presented in Figure 5.5 as fold-increases of RLU over baseline values (FIBL).

Cells transfected only with the reporters showed no evidence of NF-κB activation after incubation with either $E. \text{coli}$ LPS at a concentration of 10 ng/ml ($\text{FIBL} = 1.24 \pm 0.5$, n=4, p=0.65), $R. \text{galegae}$ LPS up to a concentration of 10 µg/ml ($\text{FIBL} = 1.02 \pm 0.08$, n=3, p=0.22) or $R. \text{Sin-1}$ LPS up to a concentration of 10 µg/ml ($\text{FIBL} = 1.03 \pm 0.09$, n=3, p=0.18). Increasing the concentration of $E. \text{coli}$ LPS to 100 ng/ml resulted in a 4.35 fold-increase of RLU over baseline
in one experiment, but in 2 other experiments did not alter the response to *E. coli* LPS (FI_{BL}=0.86 and 1.06, respectively). In summary, HEK293 cells that were not transfected with receptor proteins did not respond to rhizobial LPS at any of the concentrations tested, but did respond slightly to high concentrations of *E. coli* LPS in one experiment. In contrast to LPS stimulation, cells responded strongly to recombinant human TNF (FI_{BL}= 95.81 ± 88.35, not included in figure).

HEK293 cells transfected with human receptor plasmids and stimulated with 10 ng/ml *E. coli* LPS showed a 33.12 ± 19.99 fold-increase of RLU over baseline (n=7, p<0.0001). RLU did not significantly increase in response to *R. galegae* LPS at a concentration of 0.1 µg/ml (FI_{BL}= 3.65± 3.44, n=5, p=0.41), but did increase in response to 1 µg/ml *R. galegae* LPS (FI_{BL}= 10.76± 7.97, n=8, p=0.002). The fold-increase of RLU over baseline in response to 10 µg/ml of *R. galegae* LPS was 6.1 ± 3.73 (n=4), however, this increase was not statistically significant (p=0.63). Response to *R. Sin-1* LPS was not significant at any of the concentrations tested (FI_{BL}= 1.23± 0.56 at 0.1 µg/ml [n=5, p=0.84], 3.23 ± 2.56 at 1 µg/ml [n=8, p=0.59] and 2.68 ± 2.44 at 10 µg/ml [n=4, p=0.79]).

HEK293 cells transfected with equine receptor plasmids and stimulated with 10 ng/ml *E. coli* LPS showed a 16.67 ± 8.9 fold increase of RLU over baseline (n=9, p<0.0001). In response to *R. galegae* LPS, fold-increase of RLU over baseline was significant at concentrations of 0.1 µg/ml (FI_{BL}= 7.85 ± 6.62, n=6,p<0.0001), 1 µg/ml (FI_{BL}= 10.52 ± 7.02, n=9, p<0.0001) and 10 µg/ml (FI_{BL}= 9.17 ± 2.72, n=4, p<0.0001). In response to *R. Sin-1* LPS, activation of NF-κB was significantly increased at concentrations of 1 µg/ml (FI_{BL}= 5.97 ± 4.81, n=9, p<0.0001) and 10 µg/ml (FI_{BL}= 4.2 ± 0.97, n=4, p=0.002), but not at a concentration of 0.1 µg/ml (FI_{BL}= 3.81 ± 3.43, n=6, p=0.26).
LPS-mediated activation of NF-κB in HEK 293 cells expressing equine receptor proteins requires the presence of CD14, TLR4 and MD-2

To determine whether induction of NF-κB activation by rhizobial LPS requires the entire receptor complex or can be mediated by individual receptor proteins, HEK293 cells were transfected with individual equine receptor proteins and combinations of 2 or 3 proteins. Responses of these cells to LPS from *R. galegae* and *R. Sin-1* were then determined; *E. coli* LPS served as a control stimulus. The response to all LPS compounds was significant only if all 3 receptor proteins were expressed simultaneously (Figure 5.6). Cells transfected with only TLR4 and MD-2 and incubated with *E. coli* LPS in the presence of 10% FBS showed as robust an activation of NF-κB as cells transfected with all 3 receptor proteins, while significant NF-κB activation did not occur in the presence of 0.025% serum (data not shown).

Rhizobial LPS compounds partially inhibit *E. coli* LPS-induced NF-κB activation in HEK293 cells transfected with equine or human receptor plasmids

To determine whether rhizobial LPS compounds were able to inhibit NF-κB activation in response to *E. coli* LPS, HEK293 cells transfected with human or equine receptor plasmids were incubated for one hour with different concentrations of *R. galegae* LPS or *R. Sin-1* LPS (10 ng/ml to 10 µg/ml), and then stimulated with *E. coli* LPS (10 ng/ml) for an additional 4 hours. Unstimulated cells and cells stimulated with *E. coli* LPS alone served as controls. Results of these experiments demonstrate that *R. galegae* LPS and *R. Sin-1* LPS were able to partially inhibit *E. coli* LPS-induced NF-κB activation in cells expressing both human or equine receptor proteins (Figure 5.7). Although partial inhibition appeared to occur at individual concentrations
of rhizobial LPS, however, the results varied between experiments and inhibition did not consistently occur in a dose-dependent manner.

**DISCUSSION**

In the study reported here, we compared the biological activity of two rhizobial LPS compounds in equine and human cells. We evaluated the ability of these LPS compounds to induce TNFα production in equine monocytes and Mono Mac 6 cells, a human monocytic cell line, respectively, and tested their effect on NF-κB activation in a heterologous expression system. TNFα production is a useful indicator of LPS-induced cellular activation, as it is a proximal cytokine that is produced early in the response to LPS and is responsible for many of the pathophysiological effects of endotoxin.26, 27 NF-κB translocates to the nucleus in response to LPS-activated intracellular signaling events, and is involved in upregulation of the expression of inflammatory genes such as the TNFα gene.28 NF-κB therefore also serves as an indicator of cellular activation by LPS.

Based on their ability to stimulate TNFα production in equine monocytes (Figure 5.2), LPS from *R. galegae* and *R. Sin-1* were considered low potency agonists. The differences in potency between *E. coli* LPS and the rhizobial LPS compounds were even more striking when considering the higher amount of lipid A equivalents, which represent the active portion of LPS, per mg LPS in the rhizobial compounds. This difference in lipid A content per mg LPS was likely attributable to the carbohydrate content of the preparations, as *E. coli* O55:B5 LPS represents a smooth LPS while the preparations of *R. galegae* LPS and *R. Sin-1* LPS used in this study represent rough LPS structures. In comparison to *E. coli* LPS, the efficacy of *R. galegae*
LPS to stimulate TNFα production was considered moderate, and in some experiments, this compound exhibited characteristics of a partial agonist (Figure 5.3). Variation in the efficacy of *R. galegae* LPS between experiments and, therefore, among horses that served as blood donors, may be explained by varying LPS receptor densities on monocytes of individual horses. Variation among individuals in the surface expression of TLR4 on human monocytes has been reported, and it appears reasonable to assume that the same occurs in other species. The efficacy of *R. Sin-1* LPS was more difficult to determine, because dose-response curves did not allow fitting of a maximal TNFα activity in several experiments. At high concentrations of LPS, TNFα production increased exponentially, which was not consistent with a receptor-mediated mechanism. A similar pattern was also observed in few experiments using *R. galegae* LPS. We consider this response to be a non-physiological effect of very high concentrations of LPS, however, we cannot exclude the possibility that rhizobial LPS stimulate TNFα production in equine cells by more than one mechanism.

In contrast to their effect in equine monocyte preparations, neither rhizobial LPS compound stimulated TNFα production in human Mono Mac 6 cells, and both compounds in fact inhibited TNFα production in response to *E. coli* LPS in these cells (Figure 5.1). Our findings agree with previous reports that LPS from *R. Sin-1* does not induce TNFα production in murine peritoneal macrophages, that *R. Sin-1* LPS and *R. galegae* LPS fail to stimulate TNFα production in Mono Mac 6 cells, and that *R. Sin-1* LPS inhibits TNFα production by Mono Mac 6 cells in response to *E. coli* LPS in a competitive manner.

Equilibrium binding studies in equine monocytes showed competition for binding between rhizobial LPS and radiolabeled LPS from *E. coli* K12 LCD25 (Figure 5.4), which suggests that rhizobial LPS bind to the same cellular binding proteins as enteric LPS. Consistent
with a lesser potency in the biological function assays, affinity for binding of the rhizobial LPS compounds, as assessed by the IC$_{50}$, was reduced in comparison to *E. coli* O55:B5 LPS. In previous studies, competition between *E. coli* LPS and *R. Sin-1* LPS for binding to CD14$^{31,32}$ and LBP$^{31}$ has been demonstrated. It has further been shown that competition for LPS binding to cells occurs at the level of LBP only if LBP concentrations are limiting. In our study therefore, with serum present in excess (15%) during binding experiments, competition for radioligand binding likely occurred at the level of the cellular binding sites. We assume this binding site to be CD14, and binding studies in the presence of anti-CD14 antibodies would be helpful to prove this assumption. However, equine-specific anti-CD14 antibodies are not available at this time and antibodies against CD14 from other species do not neutralize the biological effects of LPS on equine cells (unpublished observation). We have not evaluated antibodies against CD14 from other species in binding assays using equine cells.

The effects of enteric and rhizobial LPS on NF-$\kappa$B activation in HEK293 cells expressing equine LPS receptor proteins closely resembled their effects on TNF$\alpha$ production in monocyte preparations. LPS from *E. coli*, *R. galegae* and *R. Sin-1* were able to induce NF-$\kappa$B activation (Figure 5.5), and rhizobial LPS appeared to be less potent than enteric LPS. The results obtained in the heterologous expression system were therefore consistent with the agonistic effect of both *R. galegae* LPS and *R. Sin-1* LPS observed in equine monocytes. By expressing LPS receptor proteins individually and in combination, we further showed that significant NF-$\kappa$B activation by *E. coli* LPS and rhizobial LPS occurred only if all three proteins, namely CD14, TLR4 and MD-2, were expressed concurrently (Figure 5.6). Addition of serum during cell stimulation had the same effect as transfection with CD14, suggesting that soluble CD14 contained in serum could substitute for the effects of membrane CD14. Therefore, enteric and rhizobial LPS compounds
appear to effect NF-κB activation in equine cells by interaction with CD14, TLR4 and MD-2, which is consistent with the currently accepted model of cellular activation by LPS. Our results also showed, however, that *R. galegae* LPS significantly stimulated NF-κB activation in HEK293 cells expressing human LPS receptor proteins at concentrations that did not stimulate TNFα production in Mono Mac 6 cells (Figure 5.5). NF-κB activation in response to *R. Sin-1* LPS was not statistically significant, however, this may have been attributable to a lack of statistical power due to multiple comparisons. Rhizobial LPS compounds only partially inhibited NF-κB activation in HEK293 cells transfected with human LPS receptor proteins, and a similar effect was observed in cells expressing equine proteins. These results differ from those obtained when evaluating TNFα production as an indicator of cellular activation. The results of all transfection experiments must be interpreted with caution because the heterologous expression system may not adequately reflect events in native cells and direct extrapolation of observations may not be valid. For example, over-expression of receptor proteins may result in stimulation of intracellular signaling pathways that would not be observed in monocytes or monocytic cell lines such as Mono Mac 6. Our findings suggest the possibility, however, that inhibition of TNFα production by rhizobial LPS compounds in LPS-stimulated human cells is mediated only partially by suppression of NF-κB activation. TNFα production is regulated at the transcriptional, post-transcriptional and translational level. At the transcriptional level, binding of NF-κB to promoter regulatory sites is essential for upregulation of TNFα gene transcription, however, additional transcription factors may be required for full expression of the TNFα gene. Mitogen activated protein kinases are implicated in regulation of TNFα expression at the transcriptional and translational level, and their inhibition may also contribute to suppression of TNFα production. Further studies will be required to determine which, if any,
additional mechanisms may be involved in inhibition of LPS-stimulated TNFα production by rhizobial LPS compounds.

In summary, we conclude that LPS from *R. galegae* and *R. Sin-1* are endotoxin antagonists in human monocytic cells, but are agonists of low potency and at least moderate efficacy in equine monocytes. Depending on the individual horse from which monocytes are obtained, *R. galegae* LPS exhibits characteristics of a partial or a full agonist as compared to *E. coli* LPS. In equine monocytes, rhizobial LPS compounds appear to exert their biologic effects via the LPS receptor complex consisting of CD14, TLR4 and MD-2.

**REFERENCES**


Figure 5.1: LPS from *R. galegae* (▲, solid line) and *R. Sin-1* (▼, dashed line) inhibit *E. coli* LPS-induced TNFα production in Mono Mac 6 cells. Mono Mac 6 cells were incubated for one hour with rhizobial LPS and then stimulated for 6 hours with 10 ng/ml LPS from *E. coli* O55:B5. TNFα data are normalized to represent the percent of maximum TNFα production, i.e. the concentration of TNFα produced in response to *E. coli* LPS alone. The curves represent averages ± standard deviation of 5 experiments (*R. galegae* LPS) and 8 experiments (*R. Sin-1* LPS), respectively.
Figure 5.2: LPS from *R. galegae* and *R. Sin-1* are low-affinity agonists in equine monocytes. Equine monocytes were incubated with increasing concentrations of LPS from *E. coli* O55:B5 (■, solid line), *R. galegae* (▲, interrupted line) and *R. Sin-1* (▼, dashed line), respectively, and TNF activity in supernatants determined by bioactivity assay. Data are from a representative experiment. In this experiment, estimates and 95% confidence intervals for maximum TNF activity and EC₅₀ were 1039 units/ml (882.8, 1196) and 0.041 ng/ml (0.014, 0.12) for *E. coli* O55:B5 LPS, 1405 units/ml (900.7, 1909) and 487.6 ng/ml (229.4, 1036) for *R. galegae* LPS, and 1200 units/ml and 97.53 ng/ml (54.27, 175.3) for *R. Sin-1*LPS. The maximum TNF activity in response to *R. Sin-1*LPS was constrained in order to fit the curve.
Figure 5.3: LPS from *R. galegae* is a partial agonist in some horses. Monocytes were pre-incubated for one hour with *R. galegae* LPS prior to addition of 1 ng/ml *E. coli* O55:B5 LPS and stimulation for another 6 hours (■). Control cells were stimulated with 1 ng/ml *E. coli* O55:B5 LPS alone (bar), or with *R. galegae* LPS alone (△). The graph shows an individual experiment, the results of other experiments are discussed in the text.
Figure 5.4: LPS from *R. galegae* and *R. Sin-1* compete with radiolabeled *E. coli* LPS for binding to equine monocytes. Equine monocytes were incubated with [³H]LPS from *E. coli* K12 LCD25 alone and in the presence of increasing concentrations of unlabeled LPS from *E. coli* O55:B5 (■, solid line), *R. galegae* (▲, interrupted line) and *R. Sin-1* (▼, dashed line). Data are shown as percent [³H]LPS bound with 100% being the amount bound in the absence of any competitor. Estimates and 95% confidence intervals for IC₅₀ were 0.208 ng/ml (0.125, 0.346) for *E. coli* O55:B5 LPS, 2.69 ng/ml (1.189, 6.09) for *R. galegae* LPS, and 5.56 ng/ml (0.698, 44.29) for *R. Sin-1* LPS. Data are from one representative experiment, the complete results are discussed in the text.
Figure 5.5: LPS from *R. galegae* and *R. Sin-1* stimulate NF-κB activation in HEK293 cells expressing human or equine LPS receptor proteins. HEK293 cells were transfected with CD14, TLR4 and MD-2 of human or equine origin, respectively, or with reporter plasmids only (control), and stimulated with LPS at the indicated concentrations. NF-κB activation was determined by dual luciferase assay and expressed as relative light units (RLU). The data represent the fold increase (FI) of RLU over baseline, which was determined from cells incubated with culture media alone. Data represent averages ± standard deviations of at least 5 experiments (human, equine) and at least 2 experiments (control). * indicates a significant increase of RLU over the baseline.
Figure 5.6: Equine CD14, TLR4 and MD-2 are required to render HEK293 cells responsive to *E. coli* LPS and rhizobial LPS. HEK293 cells were transfected with the indicated individual plasmids or plasmid combinations of equine origin, and with luciferase reporter plasmids. Cells were stimulated for 4 hours with LPS from *E. coli* O55:B5 (10 ng/ml, top panel) or rhizobial LPS (1 μg/ml, bottom panel) and relative light units (RLU) were determined by dual luciferase assay as a measure of NF-κB activation. Results are shown as the fold-increase of RLU over baseline, which was determined by incubation of cells with media alone. Results are the mean ± standard deviation of 3 experiments for the rhizobial LPS compounds and of 5 experiments for *E. coli* LPS. * indicates a significant increase of RLU over baseline.
**Figure 5.7:** Rhizobial LPS compounds partially inhibit *E. coli* LPS-induced NF-κB activation in HEK293 cells expressing human or equine receptor proteins. HEK293 cells were transfected with human (A) or equine (B) CD14, TLR4 and MD-2 and reporter plasmids. Cells were incubated with LPS from *R. galegae* or *R. Sin-1* for one hour prior to addition of *E. coli* LPS (10 ng/ml) and incubation for an additional 4 hours. Luciferase activity as an indicator of NF-κB activation was determined by dual luciferase assay and expressed in relative light units (RLU). The data represent the relative fold-increase (FI) of RLU over baseline, which was determined from cells incubated with culture media alone. 100% is defined as the RLU of cells incubated with *E. coli* LPS alone. * indicates a significant difference from *E. coli* LPS-stimulated cells, ® indicates a significant difference from unstimulated cells. Data represent averages ± standard deviation of 3 experiments.
Table 5.1: Effect of serum concentration in the assay on TNFα production by Mono Mac 6 cells in response to LPS from *E. coli* O55:B5. Mono Mac 6 cells were incubated with *E. coli* LPS for 6 hours and TNFα in the culture medium measured by ELISA. Results are from 3 experiments. Maximal TNFα production changed significantly in response to serum deprivation (*) while the difference in EC50 was not significant.

<table>
<thead>
<tr>
<th></th>
<th>No serum</th>
<th>5% FBS</th>
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<tbody>
<tr>
<td><strong>TNF&lt;sub&gt;max&lt;/sub&gt; (pg/ml)</strong>&lt;sup&gt;*&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean ± std dev.</td>
<td>176 ± 113</td>
<td>1715 ± 456</td>
</tr>
<tr>
<td>Median</td>
<td>101</td>
<td>1594</td>
</tr>
<tr>
<td>Range</td>
<td>71 – 296</td>
<td>1331 - 2219</td>
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<tr>
<td><strong>EC&lt;sub&gt;50&lt;/sub&gt; (ng/ml)</strong></td>
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<tr>
<td>Mean ± std dev</td>
<td>0.93 ± 0.55</td>
<td>0.56 ± 0.19</td>
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<tr>
<td>Median</td>
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<td>Range</td>
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Table 5.2: TNFα production by equine monocytes cells in response to LPS from *E. coli* O55:B5, *R. galegae* and *R. Sin-1*. Monocytes were incubated with LPS for 6 hours and TNFα in the culture medium measured by bioassay. EC₅₀ of *R. galegae* and *R. Sin-1* LPS were significantly higher than EC₅₀ of *E. coli* LPS (*). See text for discussion of maximal TNFα values.

<table>
<thead>
<tr>
<th></th>
<th><em>E. coli</em> O55:B5 (n=14)</th>
<th><em>R. galegae</em> (n=10)</th>
<th><em>R. Sin-1</em> (n=5)</th>
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<tr>
<td><strong>TNFₘₐₓ (units/ml)</strong></td>
<td></td>
<td></td>
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<tr>
<td>Mean ± std dev</td>
<td>1372 ± 2293</td>
<td>463 ± 394</td>
<td>768 ± 1140</td>
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<tr>
<td>Median</td>
<td>456</td>
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<td>339</td>
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<tr>
<td>Range</td>
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<td>99 - 1405</td>
<td>138 – 2799</td>
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<tr>
<td><strong>EC₅₀ (ng/ml)</strong></td>
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</tr>
<tr>
<td>Mean ± std dev</td>
<td>0.39 ± 0.87</td>
<td>306.24 ± 446.64*</td>
<td>131.48 ± 131.09*</td>
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<tr>
<td>Median</td>
<td>0.07</td>
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<td>103.5</td>
</tr>
<tr>
<td>Range</td>
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<td>17.52 - 1495</td>
<td>30.19 – 358.6</td>
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<tr>
<td><strong>EC₅₀ (ng lipid A equiv/ml)</strong></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Mean ± std dev</td>
<td>0.0065 ± 0.015</td>
<td>9.49 ± 13.86*</td>
<td>5.39 ± 5.37*</td>
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<tr>
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<tr>
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<td>0.54 - 46.35</td>
<td>1.24 - 14.7</td>
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Table 5.3: Effect of serum concentration in the assay on TNFα production by equine monocytes in response to LPS from *E. coli* O55:B5. Monocytes were incubated with LPS from *E. coli* O55:B5 for 6 hours and TNFα in the culture medium measured by bioassay. Results are from 6 experiments. Maximal TNFα production changed significantly in response to serum deprivation (*) while the difference in EC$_{50}$ was not significant.

<table>
<thead>
<tr>
<th></th>
<th>No serum</th>
<th>5% FBS</th>
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<tbody>
<tr>
<td><strong>TNF$_{max}$ (units/ml)</strong>*</td>
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<tr>
<td>Mean ± std dev.</td>
<td>77 ± 49</td>
<td>472 ± 439</td>
</tr>
<tr>
<td>Median</td>
<td>73</td>
<td>198</td>
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<tr>
<td>Range</td>
<td>19 – 133</td>
<td>110 - 1154</td>
</tr>
<tr>
<td><strong>EC$_{50}$ (ng/ml)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean ± std dev</td>
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<td>0.73 ± 1.28</td>
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<tr>
<td>Median</td>
<td>3.16</td>
<td>0.26</td>
</tr>
<tr>
<td>Range</td>
<td>0.2 - 102</td>
<td>0.03 – 3.32</td>
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Table 5.4: Competition for binding of $[^3H]$LPS from *E. coli* K12 LCD25 to equine monocytes by unlabeled LPS from *E. coli* O55:B5, *R. galegae* and *R. Sin-1*. The table contains values for IC50 (i.e. the concentration of unlabeled LPS that inhibited binding of radioligand by 50%) for 3 experiments. Within experiments, significant differences between IC50 of rhizobial compounds and *E. coli* O55:B5 LPS were determined by comparison of the 95% confidence intervals and are indicated by *.

<table>
<thead>
<tr>
<th>Experiment</th>
<th><em>E. coli</em> O55:B5 LPS (ng/ml)</th>
<th><em>R. galegae</em> LPS (ng/ml)</th>
<th><em>R. Sin-1</em> LPS (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.71</td>
<td>5.9*</td>
<td>6.22</td>
</tr>
<tr>
<td>2</td>
<td>0.63</td>
<td>27.88*</td>
<td>14.67*</td>
</tr>
<tr>
<td>3</td>
<td>0.11</td>
<td>1.71*</td>
<td>2.57*</td>
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<tr>
<td><strong>Average ± std dev</strong></td>
<td><strong>0.48 ± 0.33</strong></td>
<td><strong>11.83 ± 14.06</strong></td>
<td><strong>7.82 ± 6.21</strong></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Experiment</th>
<th><em>E. coli</em> O55:B5 LPS (ng lipid A equiv./ml)</th>
<th><em>R. galegae</em> LPS (ng lipid A equiv./ml)</th>
<th><em>R. Sin-1</em> LPS (ng lipid A equiv./ml)</th>
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<td><strong>Average ± std dev</strong></td>
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CHAPTER 6

EVALUATION OF LIPOPOLYSACCHARIDE BINDING TO MONO MAC 6 CELLS

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ABSTRACT

Endotoxin (lipopolysaccharide, LPS) stimulates inflammatory cells via interaction with a cellular receptor complex consisting of CD14, TLR4 and MD-2. CD14 is responsible for binding of LPS, while TLR4 in conjunction with MD-2 mediates signal transduction. This report describes the results of binding studies performed in Mono Mac 6 cells, a human monocytic cell line. Using a tritiated *Escherichia coli* (*E. coli*) LPS ligand, the number of binding sites ($B_{\text{max}}$) was estimated to be $1.13 \times 10^6$ molecules/cell and the affinity constant ($K_a$) was $5.65 \times 10^{-8}$ M. Comparison of binding site occupancy and biological response to LPS provided evidence for the existence of spare receptors. Equilibrium competition experiments to compare affinities for binding of several unlabeled LPS compounds suggested an allosteric effect on LPS binding of LPS from *Rhodobacter sphaeroides* (*Rs*LPS) and LPS from *E. coli* K12 LCD25. This effect was also demonstrated in Chinese hamster ovary (CHO) cells expressing human CD14.

INTRODUCTION

Mononuclear cells respond to endotoxin (lipopolysaccharide, LPS) by up-regulating cellular functions such as the production of cytokines. LPS elicits its effects via interaction with a receptor complex consisting of CD14 (cluster differentiation antigen 14),$^1$ TLR4 (Toll-like receptor 4),$^2$ and MD-2.$^3$ CD14 is primarily responsible for LPS binding, and LPS binding and cellular activation by LPS can be inhibited with antibodies directed against CD14.$^1,^4$ TLR4 and its associated protein MD-2 represent the signal transduction portion of the receptor complex,$^3,^5$ and direct interaction between LPS and these proteins has been demonstrated by means of genetic complementation and analysis of photoactivatable binding.$^6,^7$ Aside from CD14, TLR4
and MD-2, other proteins that bind LPS include the β2-integrins,8 selectins,9 moesin,10 scavenger receptors,11 decay-accelerating factor (CD55),12 heat shock proteins,13, 14 chemokine receptor 4 and growth differentiation factor 5.14 Binding of LPS to these proteins may play a role in LPS-induced cellular activation (e.g. β2-integrins, moesin), or be involved in cellular uptake and detoxification of LPS (e.g. scavenger receptors).

Depending on the cell type, LPS binding occurs via two alternative mechanisms. Cells expressing CD14 on their surface (membrane CD14 or mCD14) bind LPS after complex formation between LPS and a serum protein, namely lipopolysaccharide binding protein (LBP).15, 16 LBP transfers LPS monomers to the cellular surface,17, 18 and greatly enhances maximal cellular binding of LPS4 as well as the potency and efficacy of LPS in functional studies in vitro.19, 20 In the presence of LBP, LPS binds to CD14 with high affinity, and values for the dissociation constant Kd of 2.7 x 10^{-8} M in Chinese hamster ovary cells expressing human CD14 (CHO-hCD14),4 and 4.89 x 10^{-8} M4 and 8.3 x 10^{-9} M21 in human THP-1 cells have been reported. Despite the importance of LBP for LPS-receptor interaction, binding of LPS to mCD14 can occur in the absence of LBP at high concentrations of LPS.22 Cells lacking mCD14, such as endothelial cells, can bind LPS after its interaction with a soluble form of CD14 (sCD14), and transfer of the sCD14-LPS complexes to the cellular surface.23 The formation of sCD14-LPS complexes is enhanced by, but not dependent on, LBP.24

Cellular binding of LPS is inhibited by antibodies directed against CD14 and LBP,4, 22, 25 and competitively between LPS compounds. Reported molar ratios of LPS:CD14 binding range from 1 to 20,4,21,26 and binding ratios higher than 1 may be the result of self-association of LPS-LBP complexes and aggregate formation of LPS on the cellular surface.27 Only a small portion of bound LPS appears to be involved in signaling, and cellular activation still occurs when 90%
of LPS binding to mCD14 is inhibited. The exact location of binding site(s) for LPS on CD14 has not been determined and the crystal structure of CD14 is unresolved. However, it has been determined that the N-terminal 151 amino acids of mCD14 and the N-terminal 152 amino acids of sCD14 represent functional LPS receptors. Further, individual amino acids or short amino acid sequences have been identified that are essential for LPS binding.

Here, we summarize the results of radioligand binding experiments performed to estimate binding parameters for LPS binding to Mono Mac 6 cells, a human monocytic cell line. In addition to saturation experiments using tritiated \textit{E. coli} LPS, we performed competition experiments to compare the affinities for binding of LPS from \textit{Rhodobacter sphaeroides} (RsLPS) and different strains of \textit{E. coli} LPS.

\section*{MATERIALS AND METHODS}

\textbf{Lipopolysaccharides:} [\textsuperscript{3}H]LPS and unlabeled LPS from \textit{E. coli} K12 LCD25, LPS from \textit{E. coli} O55:B5, and LPS from \textit{Rhodobacter sphaeroides} (RsLPS) were purchased from List Biologicals, Campbell, CA. Additional RsLPS was isolated and purified in R. Carlson's laboratory at the University of Georgia, Athens, GA. LPS from \textit{E. coli} K12 LCD25 is an Rb rough mutant LPS lacking the O-polysaccharide. Specific activity of [\textsuperscript{3}H]LPS from \textit{E. coli} K12 LCD25 was 2.08 x 10\textsuperscript{6} dpm/µg (0.94 µCi/µg) at the time of analysis, and was corrected for decay at the time experiments were performed. [\textsuperscript{3}H]LPS from \textit{E. coli} K12 LCD25-09, which was transformed to carry a gene encoding for O-polysaccharide synthesis (O:9, a mannose homopolymer), was a generous gift from R. Munford, Dallas, TX. The specific activity of this radioligand was approximately 10\textsuperscript{6} dpm/µg.
**Cells:** Mono Mac 6 cells were provided by Dr. H.W.L. Ziegler-Heitbrock (University of Munich, Germany). Cells were grown in RPMI-1640 medium with L-glutamine that was supplemented with 10% fetal bovine serum (FBS), penicillin/streptomycin and OPI supplement containing oxaloacetate, pyruvate and bovine insulin. For 48 hours prior to experiments, cells were treated with vitamin D₃ (0.01 µg/ml cells) to enhance expression of CD14. CHO-hCD14 cells were a generous gift from P. Tobias, Scripps Institute, La Jolla, CA. Cells were grown in Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 (Ham) 1:1 (DMEM/F12) with L-glutamine that was supplemented with 10% FBS and penicillin/streptomycin.

**Radioligand binding assay:** Mono Mac 6 cells were pelleted by centrifugation; CHO-hCD14 cells were harvested by trypsinization and pelleted by centrifugation. Cells were washed once in ice-cold HNE buffer (20 mM HEPES pH 7.4, 150 mM NaCl, 1 mM EDTA) and incubated in SEBDEF buffer (20 mM HEPES pH 7.4, 150 mM NaCl, 1 mM EDTA, 10 mM NaN₃, 2 mM NaF, 5 mM deoxyglucose, 300 µg/ml bovine serum albumin) for 30 min at 37°C, 5% CO₂ to deplete adenosine triphosphate (ATP) and prevent receptor and ligand internalization during experiments. At the end of the incubation, cells were centrifuged and suspended in serum-free culture medium containing 10 mM NaN₃, 2 mM NaF, 5 mM deoxyglucose, 300 µg/ml bovine serum albumin (RPMI-B for Mono Mac 6 cells, DMEM/F12-B for CHO-hCD14 cells), and aliquots of 10⁶ cells were added to microcentrifuge tubes. Total assay volume was 0.5 ml. [³H]LPS was sonicated for 60 sec and diluted in RPMI-B or DMEM/F12-B depending on the cell type used. For competition experiments, [³H]LPS was incubated with unlabeled competitor in the presence of FBS (final concentration in assay 15%) for 30 min at room temperature prior to addition to cells. Cells were incubated with LPS at 37°C on an elliptical shaker, and binding reactions were stopped by addition of 750 µl ice-cold HNE buffer followed immediately by
centrifugation (15,000 rpm, 2 min). Supernatants were removed and cell pellets washed once in ice-cold HNE buffer and then lysed in scintillation fluid (ScintiVerse®, Fisher Scientific, Fair Lawn, NJ). Radioactive counts of cell-bound [³H]LPS were determined by liquid scintillation counting using a LS 6500 scintillation counter (Beckman Coulter Inc.). All assays were performed with duplicate or triplicate samples.

**Cell stimulation and TNFα assay:** Aliquots of 0.5 x 10⁶ Mono Mac 6 cells/tube in complete culture medium were added to 12 x 75 mm polypropylene tubes for stimulation with LPS. Cells were incubated with LPS from *E. coli* O55:B5 for 6 hours at 37°C, 5% CO₂, and then pelleted by centrifugation and supernatants collected for tumor necrosis factor α (TNFα) assay. TNFα concentration in supernatants was measured using a biotinylated enzyme-linked immunosorbent assay (ELISA) as described previously.³⁴

**Data analysis:** Binding curves and TNFα response data were fit using GraphPad Prism® software (Version 2.0, GraphPad Software, Inc., San Diego, CA). For time course evaluation of binding, the amount of cell-bound [³H]LPS was plotted against time and the curves fit using the function \( B_{\text{max}} = B_{\text{max}1} \cdot (1 - \exp(-k_1 \cdot [\text{ligand}])) + B_{\text{max}2} \cdot (1 - \exp(-k_2 \cdot [\text{ligand}])) \). For saturation experiments, the amount of cell-bound [³H]LPS was plotted against the free ligand concentration and the curves fit using the hyperbolic function \( [\text{bound ligand}] = (B_{\text{max}} \cdot [\text{free ligand}])/K_d + [\text{free ligand}] \), with \( B_{\text{max}} \) being the number of binding sites. Data were also transformed using Scatchard analysis. For competition experiments, the amount of cell-bound [³H]LPS was plotted against the concentration of unlabeled competitor and curves were fit using the function \( [\text{bound ligand}] = [\text{minimum bound}] + ([\text{maximum bound}] - [\text{minimum bound}])/1 + 10^{([\text{competitor}]-\log EC_{50})} \). Values for IC₅₀ (i.e. the LPS concentration inhibiting radioligand binding by 50%) were derived from competition curves. Competition data were transformed for Scatchard analysis by
accounting for a theoretical diluting effect of unlabeled competitor on the specific activity of radioligand in each reaction: adjusted specific activity = (specific activity x [radioligand]) / ([radioligand] + [unlabeled ligand]). The amount of cell-bound LPS at each concentration of unlabeled competitor was then re-calculated using the number of cell-associated counts and the adjusted specific activity of the radioligand. Dose-response data for TNFα were fit using the function \( \text{TNF}\alpha = \text{minimum TNF}\alpha + (\text{maximum TNF}\alpha - \text{minimum TNF}\alpha)/(1 + 10^{(\log \text{EC}_{50} - X)}) \), with \( X \) being the logarithm of LPS concentration.

RESULTS

**Influence of incubation time on LPS binding**

Figure 6.1 demonstrates the effect of incubation time on radioligand binding. Mono Mac 6 cells were incubated with radioligand ([\(^3\)H]LPS from \( E. coli \) K12 LCD25) at a concentration of 8 x 10\(^{-9}\) M for 1, 2, 5, 10, 15, 20, 30, 40, 60 and 75 min, respectively, and reactions terminated by addition of cold buffer and centrifugation. Non-specific binding of radioligand was defined as binding in the presence of an unlabeled competitor (LPS from \( E. coli \) O55:B5 at a concentration of 4.5 x 10\(^{-6}\) M). Non-specific binding increased linearly with time, while the data for total and specific binding were best fit using a two-phase exponential association function as described in the materials and methods section. From the curve, maximal binding of radioligand LPS (\( B_{\text{max}} \)) was 1.358 ng/10\(^6\) cells (0.34 pmol/10\(^6\) cells) for the first phase (\( B_{\text{max1}} \)), and 6.638 ng/10\(^6\) cells (1.66 pmol/10\(^6\) cells) for the second phase (\( B_{\text{max2}} \)). The association constant \( k \) was 0.945/min for the first (\( k_1 \)) and 0.0017/min for the second phase (\( k_2 \)), and half-life (T 1/2) was 0.734 min for the first (T 1/2\(_1\)) and 412.2 min for the second phase (T 1/2\(_2\)).
Determination of $B_{\text{max}}$ and $K_d$ in equilibrium saturation experiments

We performed equilibrium saturation experiments using $[^3\text{H}]$LPS from *E. coli* K12 LCD25-O9. Mono Mac 6 cells were incubated with increasing concentrations of radioligand (0.3125 µg/ml to 5 µg/ml) for 60 min and reactions stopped by addition of cold buffer and centrifugation. Non-specific binding was defined as binding in the presence of unlabeled *E. coli* O55:B5 LPS (10^{-5} M). Binding data are presented as a saturation isotherm and a Scatchard analysis (Figure 6.2). Because the exact molecular weight of this radioligand was not known, we used the molecular weight of LPS from *E. coli* O55:B5, another smooth LPS, for calculations. Based on the saturation isotherm, $B_{\text{max}}$ for specific binding was 2.25 pmol/10^6 cells (1.35 x 10^6 molecules/cell) and $K_d$ was 5.56 x 10^{-8} M. Repetition of the experiment with a different batch of cells yielded a $K_d$ of 5.73 x 10^{-8} M, and a $B_{\text{max}}$ of 1.51 pmol/10^6 cells (0.91 x 10^6 molecules/cell). Differences between parameter estimates in the two experiments were not significant as determined by comparison of the 95% confidence intervals. Scatchard analysis was consistent with binding to one site.

Allosteric effect of *E. coli* LPS K12 LCD25 and RsLPS on radioligand binding

We performed equilibrium competition experiments to obtain IC_{50} values for LPS from *E. coli* O55:B5 and RsLPS. We also used LPS from *E. coli* K12 LCD25 as a competitor, which represents the unlabeled form of the radioligand used in these assays. Mono Mac 6 cells were incubated for 60 min with a fixed concentration of radiolabeled ligand ($[^3\text{H}]$LPS from *E. coli* K12 LCD25) in the presence of increasing concentrations of unlabeled competitors (30 ng/ml to 50-100 µg/ml). Figure 6.3 presents the normalized results of these experiments, where 100% binding is defined as binding of radioligand in the absence of competitor. Because the molecular
weight of RsLPS was not known, concentrations of LPS are given in (µg/ml). LPS from *E. coli* O55:B5 displaced the radioligand in a simple competitive manner with an IC$_{50}$ of 1.2 µg/ml. When either RsLPS or LPS from *E. coli* K12 LCD25 was used as the competitor, however, competition curves were not consistent with simple displacement but instead suggested an allosteric effect of these compounds on radioligand binding. Allosteric interaction may be defined as one that occurs between two topographically distinct binding sites on the same receptor complex.³⁵ By interaction with an allosteric site, a ligand may modulate ligand binding to the orthosteric site, i.e. the site that is recognized by the receptor agonist.³⁵ In our experiments, lower concentrations of RsLPS or LPS from *E. coli* K12 LCD25 (below approximately 3 µg/ml) enhanced binding of the radioligand, whereas competition for radioligand binding was observed at higher concentrations of competitor. The bottom plateau of all curves was similar, suggesting that overall, RsLPS and LPS from *E. coli* K12 LCD25 competed for radioligand binding to a similar extent as LPS from *E. coli* O55:B5. For RsLPS, similar results were obtained using commercially available LPS or LPS isolated in Dr. R. Carlson’s laboratory at the University of Georgia (data not shown), although the effect was more pronounced using the commercially available LPS.

Because these competition curves suggested an allosteric effect of RsLPS and LPS from *E. coli* K12 LCD25 on radioligand binding, we evaluated the effect of these compounds on competition for radioligand binding by other LPS ligands. The compounds were added to competition experiments at a concentration that, by itself (Figure 6.3), induced maximal binding of the radioligand. The results are presented in Figures 6.4, 6.5 and 6.6. Figure 6.4 shows the effect of RsLPS (3 µg/ml) on competition for radioligand binding by increasing concentrations of LPS from *E. coli* O55:B5. In the absence of RsLPS, maximal binding of radioligand reached
0.282 pmol/10^6 cells, minimal binding was 0.061 pmol/10^6 cells, and IC_{50} of LPS from \textit{E. coli} O55:B5 was 1.1 \times 10^{-7} \text{ M}. In the presence of \textit{RsLPS}, maximal binding was 1.153 pmol/10^6 cells, minimal binding was 0.032 pmol/10^6 cells, and IC_{50} was 6.68 \times 10^{-8} \text{ M}. Comparison of the 95% confidence intervals showed that maximal radioligand binding differed significantly between the two curves, whereas minimal binding and IC_{50} values did not. Therefore, \textit{RsLPS} increased the maximal binding of radioligand, which was consistent with the results shown in Figure 6.3, but did not affect the affinity of \textit{E. coli} O55:B5 LPS or its efficacy in inhibiting radioligand binding.

Figure 6.5a shows the effect of \textit{RsLPS} (3 \mu g/ml) on competition for radioligand binding by increasing concentrations of LPS from \textit{E. coli} K12 LCD25. In the absence of \textit{RsLPS}, maximum binding of radioligand was reached at a concentration of competitor of approximately 2.5 \times 10^{-7} \text{ M}. Analysis of a partial curve (excluding data points prior to maximal radioligand binding) yielded a maximum of 1.244 pmol/10^6 cells, a bottom plateau of zero, and an IC_{50} of 10.2 \times 10^{-7} \text{ M}. In the presence of \textit{RsLPS}, the curve was consistent with a simple competition for binding; maximal binding was 1.217 pmol/10^6 cells, the bottom plateau was zero and IC_{50} was 9.4 \times 10^{-7} \text{ M}. Comparison of 95% confidence intervals showed no significant difference between maximal binding reached in the absence or presence of \textit{RsLPS}, or between the values for IC_{50} (using analysis of the partial curve for data in the absence of \textit{RsLPS}). \textit{RsLPS}, therefore, increased binding of radioligand at low concentrations of LPS from \textit{E. coli} K12 LCD25, but did not affect maximal binding of radioligand or the affinity of the competitor. Scatchard analysis of the data in the presence of \textit{RsLPS} is shown in Figure 6.5c and is consistent with a one-site binding event.

Figure 6.5b presents the results of the “reversed” experiment from Figure 6.5a, i.e. competition for radioligand binding by \textit{RsLPS} in the presence and absence of LPS from \textit{E. coli}
K12 LCD25 (3 μg/ml, 7.5 x 10^{-7} M). LPS from *E. coli* K12 LCD25 had a less pronounced effect than *Rs*LPS (Figure 6.5a), however, LPS from *E. coli* K12 LCD25 also increased radioligand binding at low concentrations of *Rs*LPS and changed the shape of the curve towards a simple one-site competition curve. To evaluate whether our findings were limited to Mono Mac 6 cells, we performed similar experiments in a different cell line, namely CHO-hCD14 cells. Results were similar to those obtained in Mono Mac 6 cells and are presented in Figure 6.6.

**Evidence for spare receptors on Mono Mac 6 cells**

To investigate whether spare receptors are present on Mono Mac 6 cells, we compared the effect of LPS concentration on the occupancy of binding sites and the stimulation of cellular responses, respectively (Figure 6.7). Occupancy data were derived from the saturation experiment shown in Figure 6.2, using [3H]LPS from *E. coli* K12 LCD25-O9 as the radioligand. Because an unlabeled form of this radioligand was not available for cell stimulation assays, TNFα production as an indicator of cellular stimulation was measured in response to LPS from *E. coli* O55:B5. Comparison of these LPS compounds seemed valid because both represent a smooth form *E. coli* LPS and had similar affinities for binding to Mono Mac 6 cells in our experiments. Estimates for the affinities of the two compounds were 10^{-7} M for LPS from *E. coli* O55:B5 (based on IC_{50} in competition experiments, Figure 6.3 and 6.4), and 5.65 x 10^{-8} M for [3H]LPS from *E. coli* K12 LCD25-O9 (determined in saturation experiments). Figure 6.7 demonstrates that maximal TNFα production occurred at a very low occupancy of binding sites (approximately 10% as indicated by the dashed line). This discrepancy between occupancy and response suggests the presence of spare receptors.
DISCUSSION

The purpose of this study was to quantitatively evaluate LPS binding to Mono Mac 6 cells, a cell line that is used routinely as a representative of human monocytic cells, and to compare binding affinities between *E. coli* LPS and *Rs* LPS in competition experiments. Lipid A from *Rhodobacter sphaeroides* is a potent endotoxin antagonist in human and murine cells, and we have previously determined that *Rs*LPS is an antagonist in Mono Mac 6 cells as shown by its ability to inhibit TNFα production in response to *E. coli* LPS.\(^3^6\) It has been suggested that LPS antagonists act in a competitive manner,\(^3^7\) which prompted us to evaluate the ability of *Rs*LPS to compete with *E. coli* LPS for binding to Mono Mac 6 cells.

Due to practical constraints, different radioligands were used in this study. Equilibrium saturation experiments were performed with a tritiated LPS from *E. coli* K12 LCD25-O9, while competition experiments were performed with tritiated LPS from *E. coli* K12 LCD25. *E. coli* K12 LCD25 produces a rough LPS of Rb chemotype, (i.e. the LPS lacks the O-saccharide portion and part of the outer core), while *E. coli* K12 LCD25-O9 represents the same bacterial strain after transformation with a gene encoding for synthesis of a mannose homopolymer (O:9). *E. coli* K12 LCD25-O9 therefore produces LPS carrying an O-chain, however, substitution with the O-polysaccharide may be incomplete (R. Munford, personal communication).

A time course for LPS binding to Mono Mac 6 cells was characterized by a very rapid first phase and a prolonged second phase, and was consistent with the presence of more than one binding site (Figure 6.1). The observed rapid phase of LPS binding is consistent with a previous report of rapid binding of LPS to CD14,\(^4\) while the second, prolonged phase was unexpected.

Several proteins other than CD14 bind LPS and might have contributed to the second phase of binding in this experiment. Alternatively, the second phase may represent accumulation of LPS
aggregates on the cellular surface, direct intercalation of LPS into the cell membrane, or incomplete inhibition of ligand internalization.

Equilibrium saturation experiments using \[^{3}H\]LPS from *E. coli* K12 LCD25-O9 as the radioligand yielded estimates for $B_{\text{max}}$ of $1.13 \times 10^6$ molecules/cell and for $K_d$ of $5.65 \times 10^{-8}$ M (average of two experiments). We did not have an independent estimate of the receptor density of CD14 on Mono Mac 6 cells, however, an estimate of $6.8 \times 10^5$ CD14 molecules/cell was obtained in vitamin D$_3$-stimulated THP-1 cells, another human monocytic cell line.\(^4\) Assuming a similar receptor density on Mono Mac 6 cells, a ratio for binding of LPS to CD14 of 1.7 was calculated, which compares favorably with the 1:1 binding stoichiometry of other reports.\(^2\) A slightly higher than 1:1 ratio in our experiments might be explained by the fact that equilibrium experiments were carried out with a incubation time of 60 min. According to our time course experiments (Figure 6.1), binding to more than one site would be measured at this time. Additional experiments using shorter incubation times, and an independent determination of the number of CD14 molecules on Mono Mac 6 cells will be required to definitively determine the ratio of LPS:CD14 binding in these cells.

Because LPS from *E. coli* O55:B5 and RsLPS were not available in radiolabeled form, the affinity of these ligands was determined in equilibrium competition experiments (Figure 6.3). We further performed self-displacement experiments using unlabeled LPS from *E. coli* K12 LCD25 as the competitor (Figure 6.3). Competition for binding of a radioligand allows determination of $IC_{50}$ (i.e. the concentration of unlabeled competitor that inhibits radioligand binding by 50%), which can be compared between compounds as a measure of affinity. In self-displacement experiments, where radioligand and unlabeled competitor are of identical structure, $K_d$ is calculated as $K_d = IC_{50} - [\text{radioligand}]$.\(^3\) The results of our competition experiments
suggested an allosteric effect of RsLPS and LPS from *E. coli* K12 LCD25 on radioligand binding, while LPS from *E. coli* O55:B5 competed for binding in a simple, dose-dependent manner. RsLPS and LPS from *E. coli* K12 LCD25 exhibited the allosteric effect at concentrations below approximately 3 µg/ml, but competed for binding of the radioligand at higher concentrations. This suggested that RsLPS and LPS from *E. coli* K12 LCD25 bound to both an allosteric and the orthosteric site in a concentration-dependent manner, while LPS from *E. coli* O55:B5 bound only to the orthosteric site. The allosteric effect of RsLPS and LPS from *E. coli* K12 LCD25 was further demonstrated by their effect on the competition curves of other unlabeled ligands (Figures 6.4-6.6). At a concentration of 3 µg/ml, both LPS compounds enhanced binding of the radioligand without affecting the affinity of other competing LPS. Because their effect on maximal binding of radioligand was not additive (Figure 6.5a, 6.5b and 6.6), we further concluded that RsLPS and LPS from *E. coli* K12 LCD25 bind to the same allosteric site. The fact that experiments were repeatable and yielded similar results in two different cell lines (Mono Mac 6 and CHO-hCD14), strongly suggests that our findings were not attributable to an experimental artifact. However, because we did not re-purify LPS prior to experiments and contamination of LPS preparations with biologically active lipoproteins has been reported,39 we cannot exclude the possibility that the allosteric effect observed in our experiments was caused by an impurity of the LPS preparations and not by LPS itself.

To our knowledge, allosteric regulation of LPS binding has not been reported previously. Quantitative evaluation of binding has focused on CD14, and several regions of CD14 have been identified that are crucial for LPS binding.29-33 On the basis of our experiments, we cannot speculate on the exact location of an allosteric binding site within the LPS receptor complex; however, the allosteric site and the orthosteric site must be located on the same protein or on
closely associated proteins. CD14 contains binding sites for various ligands other than LPS, and might also contain an allosteric site. As LPS interacts with all proteins comprising the receptor complex, it is further possible that allosteric and orthosteric sites are located on different proteins that come into close contact during LPS binding. We cannot comment on the functional consequences of an allosteric regulation of LPS binding at this time, and appropriate experiments will need to be designed to investigate this issue.

Previously, Kitchens et al. provided evidence for the existence of spare receptors on THP-1 cells. We compared TNFα production as an indicator of receptor function with binding data obtained from saturation experiments and determined that maximal TNFα production was achieved at very low receptor occupancy (Figure 6.7). With the caveat that different strains of E. coli LPS were used for functional and binding assays, respectively, we therefore provide evidence supporting the existence of spare receptors on Mono Mac 6 cells. The presence of spare receptor is to be expected because LPS-induced cellular activation occurs in the presence of minute amounts of LPS, and LPS-induced signaling is not inhibited even when LPS binding is prevented to a large extent.

REFERENCES


Figure 6.1: Time course of LPS binding to Mono Mac 6 cells. Cells were incubated with 
[\(^3\)H]LPS from *E. coli* K12 LCD25 (8 x 10^{-9} M) for 1, 2, 5, 10, 15, 20, 30, 40, 60 and 75 min, 
respectively; non-specific binding was determined in the presence of unlabeled LPS from *E. coli* 
O55:B5 (4.5 x 10^{-6} M). TB = total binding (interrupted line), SB = specific binding (solid line), 
NS = non-specific binding (dashed line).
**Figure 6.2:** Saturation isotherm and Scatchard analysis (inset) of an equilibrium saturation experiment. Mono Mac 6 cells were incubated with $[^3]$H]LPS from *E. coli* K12 LCD25-O9 at increasing concentrations for 60 min; non-specific binding was determined in the presence of unlabeled *E. coli* O55:B5 LPS ($10^{-5}$ M). TB = total binding (interrupted line), SB = specific binding (solid line), NS = non-specific binding (dashed line).
Figure 6.3: Competition for binding of $[^3]$HLPS from *E. coli* K12 LCD25 by unlabeled LPS from *E. coli* O55:B5 (solid line), *E. coli* K12 LCD25 (dashed line) and *Rs* LPS (interrupted line). The percent radioligand bound is plotted against the concentration of competitor; 100% binding is defined as binding of radioligand in the absence of competitor.
**Figure 6.4:** Competition for radioligand binding by LPS from *E. coli* O55:B5 in the presence and absence of RsLPS. Mono Mac 6 cells were incubated for 60 min with radioligand ([3H]LPS from *E. coli* K12 LCD25; 7.25 x 10⁻⁹ M) and increasing concentrations of unlabeled LPS from *E. coli* O55:B5 in the presence (dashed line) and absence (solid line) of RsLPS (3 µg/ml).
Figure 6.5a: Competition for radioligand binding by LPS from *E. coli* K12 LCD25 in the presence and absence of RsLPS. Mono Mac 6 cells were incubated for 60 min with radioligand ([³H]LPS from *E. coli* K12 LCD25; 7.25 x 10⁻⁹ M) and increasing concentrations of unlabeled LPS from *E. coli* K12 LCD25 in the presence (dashed line) and absence (solid line) of RsLPS (3 µg/ml).
Figure 6.5b: Competition for radioligand binding by RsLPS in the presence and absence of LPS from *E. coli* K12 LCD25. Mono Mac 6 cells were incubated for 60 min with radioligand ([³H]LPS from *E. coli* K12 LCD25; 7.25 x 10⁻⁹ M) and increasing concentrations of unlabeled RsLPS in the presence (dashed line) and absence (solid line) of LPS from *E. coli* K12 LCD25 (3 µg/ml).
Figure 6.5c: Scatchard analysis of the experiment shown in Figure 6.5a. Data were from competition experiments using LPS from *E. coli* K12 LCD25 LPS in the presence of *R. sphaer.* LPS (3 µg/ml).
Figure 6.6: Competition for radioligand binding by LPS from *E. coli* K12 LCD25 in the presence and absence of RsLPS in CHO-hCD14 cells. CHO-hCD14 cells were incubated for 60 min with radioligand ([³H]LPS from *E. coli* K12 LCD25; 3.875 x 10⁻⁹ M) and increasing concentrations of unlabeled LPS from *E. coli* K12 LCD25 in the presence (dashed line) and absence (solid line) of RsLPS (3 µg/ml) (A). Scatchard analysis of data obtained in the presence of RsLPS is shown in panel B.
Figure 6.7: Evidence for the existence of spare receptors on Mono Mac 6 cells. The effect of LPS concentration on TNF$\alpha$ production and LPS binding, respectively, in Mono Mac 6 cells is compared. TNF$\alpha$ production was measured in response to *E. coli* O55:B5; binding data are from the saturation experiment shown in Figure 6.2 using $[^3]$H]LPS from *E. coli* K12 LCD25-O9 as the radioligand. Maximal TNF production is reached at concentrations of LPS, which saturate only a small fraction (approximately 10%) of receptors (dashed vertical line).
CONCLUSION

The main purpose of the studies reported here was to investigate the biological activities of different structurally atypical lipopolysaccharides (LPS) in order to determine their potential value as endotoxin antagonists in horses. Horses are prone to the development of endotoxemia as they are very sensitive to the effects of endotoxin, and suffer commonly from diseases associated with an exposure to Gram-negative bacteria.¹ Translocation from the intestinal lumen may be of particular importance in horses because acute gastrointestinal disease (colic) occurs with a high prevalence in this species. Conditions that compromise the intestinal barrier function, such as severe inflammation or strangulating obstructions that reduce mucosal blood flow, may allow increased translocation of endotoxin to occur. Although immune defense mechanisms, specifically the hepatic mononuclear phagocytic system, are set up to recognize and clear small amounts of translocated endotoxin, a sudden onslaught of endotoxin and/or bacteria can result in a “spill over” of endotoxin into the systemic circulation and activation of systemic inflammatory cascades. In equine medicine, treatment options that address the cause of endotoxemia (i.e. the interaction between endotoxin and inflammatory cells) are currently limited and include antimicrobial treatment of underlying Gram negative infections, immune therapy using antibodies directed against specific regions of the LPS molecule or endotoxin-binding drugs such as polymyxin B.² Endotoxin antagonists offer another means of preventing endotoxin interaction with inflammatory cells and may therefore complement available treatments. The therapeutic value of endotoxin antagonists is probably restricted to the very early stages of endotoxemia,
before the inflammatory response has entered the stage of being perpetuated by the effects of inflammatory mediators rather than endotoxin itself.

In our studies, we investigated the biological activities of LPS from *Rhodobacter sphaeroides* (*Rs*LPS), *Rhizobium galegae* (*R. galegae*) and *Rhizobium Sin-1* (*R. Sin-1*). We used TNFα production by equine monocytes as an indicator of cellular response, and compared equine responses to those of human Mono Mac 6 cells. The comparison to human cells served two purposes. First, it allowed us to detect species-specific differences in the response to the LPS compounds. Second, and just as importantly, it provided a method of “quality control” in our assays. Previous investigators have demonstrated that lipoprotein contamination of LPS preparations was responsible for erroneous results (e.g., suggesting cellular activation by LPS via TLR2 instead of TLR4) and that degradation of LPS compounds over time could result in an alteration of biological activity. We did not re-purify commercially available LPS compounds used in this study, however, we feel that the consistent recognition of all three LPS compounds as agonists in human cells provides sufficient proof that our LPS preparations retained their biological activity and that our results in equine cell preparations were due to the recognition by cells of LPS rather than of other immune stimulatory molecules. We cannot exclude the formal possibility that contaminants with an equine-specific stimulatory capacity were responsible for our results, however, this appears to be quite unlikely.

In the first study of this report, we evaluated the effects of *Rs*LPS on equine and human cells; the third study describes our findings with regards to the rhizobial LPS compounds. We determined that *Rs*LPS and LPS from *R. galegae* and *R. Sin-1* were agonists in equine cells while being antagonists in human cells. The efficacy of LPS from *R. galegae* relative to *E. coli* LPS varied among individual horses and LPS from *R. galegae* was a partial agonist in individual
horses. RsLPS and LPS from R. Sin-1 were considered full agonists. Potencies varied considerably among the compounds, with RsLPS being as potent as E. coli LPS and rhizobial LPS having a significantly reduced potency. The central conclusion from these experiments is that LPS analogues based on the structures of RsLPS, R. galegae LPS and R. Sin-1 LPS cannot be expected to have therapeutic value in horses and may indeed be detrimental. An additional implication is that any endotoxin antagonists that may become available for the treatment of human patients need to be evaluated for use in horses using equine-specific experimental systems, as they cannot be expected to have the same effects in equine cells as they might have in human cells.

Given our results obtained in monocyte stimulation assays, we performed additional experiments to answer the question of how RsLPS, R. galegae LPS and R. Sin-1 LPS stimulate equine cells, and how signaling events in equine cells compare to those in human cells. The results are presented in the second and third study of this report. Using transfection experiments in HEK293 cells, we first investigated the role of CD14, TLR4 and MD-2 in the cellular response to RsLPS and rhizobial LPS compounds. Using RsLPS as the compound that most drastically demonstrated species-specific differences between equine and human cells, we further investigated the effect that species origin of each individual receptor protein had on the cellular phenotype, i.e. the cells' recognition of RsLPS as an agonist or antagonist. Because transfected HEK293 cells do not produce TNFα in response to LPS, we used a dual luciferase assay to determine cellular activation. This method allowed us to measure activation of NF-κB, a transcription factor that is prominently activated in response to LPS and that is centrally involved in the up-regulation of TNFα expression following an LPS stimulus. It is tempting to interpret NF-κB activation in transfected HEK293 cells by LPS compounds that stimulate TNFα.
production in monocytic cells as evidence for a causal relationship between NF-κB activation and TNFα production. However, this connection remains to be proven for each of the atypical LPS compounds. Interpretation of all experiments using transfected cells should further take into account that heterologous expression systems are artificial systems, which may not adequately represent the events in primary monocytes and/or monocytic cell lines. Results obtained in experiments using transfected cells should, therefore, be confirmed in native cells whenever possible. NF-κB activation in native cells may be evaluated by means of electrophoretic mobility shift assays or by transfection of reporter enzyme plasmids into these cells. Given this caveat, our transfection experiments yielded several interesting results, which warrant further investigation and confirmation.

Using HEK293 cells expressing human or equine receptor proteins, we showed that the effects of RsLPS on NF-κB activation mirrored its effects on TNFα production in monocytic cells. In other words, RsLPS stimulated NF-κB activation in cells expressing equine receptor proteins, while it inhibited endotoxin-induced NF-κB activation in cells expressing human receptor proteins. These findings suggest that RsLPS exerts its effects at the level of NF-κB activation, and are consistent with this compound being a competitive inhibitor of NF-κB activation and TNFα production in human cells.

Surprisingly, the recognition of RsLPS by HEK293 cells expressing equine receptor proteins occurred independent of CD14. It has so far been understood that CD14 acts as the primary binding protein for LPS, and that transfer of LPS by CD14 to the TLR4/MD-2 complex is required for signal transduction to occur, however, CD14-independent cellular stimulation at high concentrations of LPS has been reported previously. It is therefore possible that the observed activation of HEK293 cells expressing equine TLR4/MD-2 but not CD14 was due to
relatively high concentrations of RsLPS. This issue warrants further investigation, however, we did not observe stimulation of cells in the absence of CD14 by an equivalent concentration of E. coli LPS, suggesting that the response to RsLPS was inherently different and not an effect of LPS concentration. To date, it is difficult to investigate the function of individual receptor proteins in native equine cells because equine-specific antibodies against these proteins are not available. Should our findings prove consistent in future studies, the possibility of an alternative LPS binding protein present on the cellular surface of equine cells should be investigated.

Similar to RsLPS, the effects of rhizobial LPS compounds on HEK293 cells expressing equine receptor proteins were consistent with their effects on TNFα production in native cells. We demonstrated that rhizobial LPS activated NF-κB, and that this activation was dependent on the presence of CD14, TLR4 and MD-2. Therefore, rhizobial LPS appear to exert their effects in equine cells via the same receptor complex as enteric LPS, a hypothesis that is supported by the results of binding experiments, in which rhizobial LPS competed with enteric LPS for cellular binding sites. As mentioned previously, the causal relationship between NF-κB activation and TNFα production in equine cells in response to RsLPS and rhizobial LPS remains to be proven. In contrast, the effects of rhizobial LPS on NF-κB activation in HEK293 cells expressing human receptor proteins were not consistent with their effects on TNFα production. R. galegae LPS effected significant NF-κB activation in these cells and neither rhizobial LPS compound completely inhibited NF-κB activation in response to enteric LPS. Activation of NF-κB was dependent on the presence of CD14, TLR4 and MD-2 and was therefore not due to a non-specific effect of rhizobial LPS compounds on HEK293 cells. Taking into account the caveat regarding the use of heterologous expression systems, these findings suggest the possibility that rhizobial LPS inhibit TNFα production in human cells by mechanisms other than competitive
antagonism. Regulation of TNFα production is complex and possible targets for regulation by rhizobial LPS compounds that should be investigated include transcription factors other than NF-κB, as well as post-transcriptional and translational mechanisms. An intriguing, although mostly speculative, alternative explanation of our results is based on reports that *R. Sin-1* LPS stimulates CD14 expression in murine bone marrow cells via TLR2 in combination with TLR6. Given these results and the increasing amount of information regarding “cross talk” among Toll-like receptors, it is at least possible that rhizobial LPS require additional or alternative receptors than CD14, TLR4 and MD-2 to exert an antagonistic effect.

In the second study of this report, we demonstrated that the combination of TLR4 and MD-2 is responsible for the equine-specific recognition of *R* sLPS as an agonist. Equine TLR4 in conjunction with human MD-2 (and, vice versa, human TLR4 in conjunction with equine MD-2) conferred responsiveness to *E. coli* LPS on HEK293 cells in the presence of CD14 of either species, thereby proving that the combinations as such yielded functional receptors. In contrast, only cells expressing both equine TLR4 and MD-2 responded to *R* sLPS by activation of NF-κB. The species origin of CD14 was inconsequential for the recognition of *R* sLPS as an agonist, which was consistent with previous reports. These results support the conclusion that the “recognition site” for *R* sLPS on the equine LPS receptor complex involves regions of both TLR4 and MD-2. It remains to be investigated whether *E. coli* LPS and *R* sLPS are recognized via the same “recognition site” and which structural features are responsible for recognition of agonists and antagonists, respectively, in different species. Site-directed mutagenesis of TLR4 and/or MD-2 proteins may be useful in identifying the role of specific amino acids in the recognition of *R* sLPS by equine receptor proteins. Previous reports have implied a role for hypervariable regions in TLR4 and certain amino acids of MD-2 in the species-specific recognition of
different LPS structures, and investigation of these sites in equine proteins is warranted. Elucidation of the three-dimensional structure of the TLR4/MD-2 complex in the future will further provide important information regarding the location and characteristics of LPS recognition sites.

It has been suggested that a conical three-dimensional shape of LPS determines its recognition as an agonist, while a cylindrical shape results in recognition as an antagonist.\textsuperscript{11} Given our results and the results of previous studies demonstrating an agonistic effect of \textit{RsLPS} in hamster cells,\textsuperscript{8} the overall validity of this concept must be put into question. It would be interesting to investigate whether the “endotoxic conformation” of LPS varies depending on the host species, or whether certain species are more restricted in their recognition of “endotoxic” LPS shapes. It appears unlikely that \textit{RsLPS} changes its shape by some species-specific mechanism, such as interaction with certain serum proteins, because its agonistic and antagonistic effects, respectively, are observed in cell culture under exclusion of the native cellular environment.

In the fourth study of this report, we investigated LPS binding to Mono Mac 6 cells. Using tritiated \textit{E. coli} LPS, we obtained estimates for the number of binding sites for enteric LPS and the affinity of enteric LPS to these binding sites. However, we could not definitively determine the ratio of LPS:receptor binding in this cell type. Independent estimation of the number of CD14 molecules on Mono Mac 6 cells as has been described for other cell types,\textsuperscript{12} may be helpful in further elucidating the mechanisms of LPS binding to these cells. An unexpected finding, which warrants further investigation, was that \textit{E. coli} K12 LCD25 LPS and \textit{RsLPS} appeared to allosterically regulate LPS binding to Mono Mac 6 cells. It will be necessary to confirm that this effect was mediated by LPS itself, e.g. by repeating the experiments after re-
purification of LPS compounds. Should the results be consistent, it would be interesting to evaluate additional LPS compounds of varying structure in order to determine structural components that mediate this allosteric effect. The functional consequence of allosteric regulation of LPS binding remains to be investigated. To this end, it will be necessary to evaluate the effect of *E. coli* K12 LCD25 LPS (which was used as the radioligand in our studies) on a parameter of cellular activation, such as TNFα production, in the presence and absence of varying concentrations of *Rs*LPS.

Lastly, comparison of binding site occupancy in saturation experiments with LPS potency in functional assays provided evidence for the existence of spare receptors on Mono Mac 6 cells. Spare receptors are a common phenomenon; spare receptors for LPS have been suggested to exist in other cell types and are likely responsible for the recognition of minute amounts of endotoxin by inflammatory cells. Spare receptors may explain findings that blockade of LPS binding to over 90% of CD14 molecules with anti-CD14 antibodies did not inhibit cellular responses. The existence of spare receptors does not, however, preclude a potential therapeutic use of competitive LPS antagonists because competition for receptor binding occurs independent of the absolute cell number.

REFERENCES


