Interactions of bacterial lipopolysaccharide (LPS) with CD14 receptors, bovine alveolar macrophages, and vascular endothelial cells

Zhengang Yang

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To the Graduate Council:

I am submitting herewith a dissertation written by Zhengang Yang entitled "Interactions of bacterial lipopolysaccharide (LPS) with CD14 receptors, bovine alveolar macrophages, and vascular endothelial cells." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Comparative and Experimental Medicine.

Phillip N. Boschsler, Major Professor

We have read this dissertation and recommend its acceptance:

David O. Slauson, Rodger C. Carroll, Timothy W.J. Olchowy

Accepted for the Council:

Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)
To the Graduate Council:

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Accepted for the Council:

Associate Vice Chancellor and Dean of The Graduate School

November 8, 1995
INTERACTIONS OF BACTERIAL LIPOPOLYSACCHARIDE (LPS)
WITH CD14 RECEPTORS, BOVINE ALVEOLAR
MACROPHAGES, AND VASCULAR ENDOTHELIAL CELLS

A
Dissertation
Presented for the
Doctor of Philosophy Degree
The University of Tennessee, Knoxville

Zhengang Yang
December, 1995
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Abstract

Monocytes, macrophages, and vascular endothelial cells (ECs) are major cellular targets of bacterial lipopolysaccharide (LPS). The activation or damage of these cells by LPS contributes to clinical sequelae in gram-negative bacteremia and endotoxemia. The manner in which LPS engages the target cells to trigger stimulation is one of the key factors in understanding the pathogenesis of gram-negative bacterial infection. The purposes of this study are to demonstrate the existence and function of bovine CD14, investigate its interaction with LPS and LPS-binding protein (LBP), and study novel pathways associated with soluble CD14 (sCD14) and membrane-associated CD14 (mCD14).

First, we describe the presence and function of an mCD14-like receptor on bovine alveolar macrophages (bAM). Immunofluorescence and flow cytometric analysis indicated specific binding of anti-human CD14 monoclonal antibodies (MAbs) to bAM. Pretreatment of bAM with phosphatidylinositol specific phospholipase C reduced the binding. Anti-CD14 MAb inhibited the binding of $^{125}$I-LPS to bAM. Bovine CD14 mRNA was detected, and was moderately upregulated by LPS stimulation. Functional assays with LPS-stimulated bAM showed that anti-CD14 MAb inhibited tissue factor expression in a chromogenic assay, and also inhibited expression of tissue factor mRNA in northern blot analysis.

Secondly, we identified mCD14 associated with bAM and also sCD14 in
bovine serum. A combination of immunoprecipitation and autoradiography detected a single band of protein with a molecular mass of approximately 49.5 kDa. A "carbohydrate-free" protein about 38-40 kDa was detected with hybrid-selected translation. In vitro translated proteins using total RNA from bAM was subjected to immunoprecipitation with anti-CD14 MAb, and a 38 kDa protein was detected. sCD14 in bovine serum was demonstrated with a western blot analysis.

In addition, bovine vascular endothelial cells (bECs) were used and the cytotoxicity mediated by LPS was measured by a chromogenic MTT assay. Recombinant soluble CD14 and purified bovine LBP were combined to determine the pathway of LPS-mediated cytotoxicity and activation of bECs. The data show that sCD14 is required for LPS activation and the LBP dramatically decreased the required amount of LPS to activate bEC in the presence of sCD14.

Finally, using the tissue factor expression assay and the ^3H-LPS binding assay, we demonstrate novel pathways taken by LPS. In addition to binding to sCD14, LPS may be able to directly bind to mCD14, although the interaction is quite weak. First, sCD14 at 37°C is able to transfer LPS to its membrane receptor on bAM in the absence of other serum factors, resulting in cell activation. This is contrary to the results obtained with addition of rsCD14 into serum-containing medium. A "three-step-efficiency" model is proposed. In the presence of both LBP and CD14, the highest level of efficiency for LPS binding
and activation is achieved. When only sCD14 or mCD14 is present, the efficiency is moderate. The direct interaction of LPS with its transducer/receptor is of quite low efficiency. This model logically explains that the effects of protein factors in both monocyte/macrophage and EC systems is quite similar, although the efficiency of the same route in each cell type may be greatly variable due to the presence or absence of molecular components.
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PART 1
INTRODUCTION

Background
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LBP, an accelerator for formation of CD14-LPS complex
CD14, a LPS receptor, but not a signal transducer
LPS signal transducer, an unknown transmembrane component
Current models for extracellular pathways of LPS
Significance of studying bovine CD14 receptors
References
**Background**

Septicemia or endotoxemia caused by gram-negative infection in modern clinical practice is still a serious issue, and is associated with a high mortality rate. Primary infection in immunocompromised patients, and especially secondary infections associated with cancer or acquired immunodeficiency syndrome (AIDS) are still a big challenge to medical doctors. The major causative factor responsible for the complicated clinical symptoms in gram-negative bacterial infection is lipopolysaccharide (LPS), a component of the outer cell wall of the bacteria. However, recent studies have shown that LPS has very limited direct actions, and acts by induction of proinflammatory cytokines and other mediators which cause the clinical signs and pathologic sequelae (1-7). It is now known that LPS-induced mononuclear phagocyte activation with synthesis and release of proinflammatory agents such as tumor necrosis factor α (TNF-α), interleukin-1 (IL-1) and interleukin-6 (IL-6), interferon (IFN), platelet-activating factor (PAF), and various eicosanoids is a key initiating event in the pathogenesis of gram-negative bacterial diseases. Furthermore, the LPS-mediated tissue factor expression on both monocyte/macrophage and vascular endothelial cells is the initiator of microvascular coagulation in gram-negative bacterial infection.

LPS may initiate signs of sepsis by acting locally on cells at specific organ sites of infection, or it may be absorbed into the bloodstream from the intestine, mammary gland, lung, and other sites to act systemically. In the
bloodstream, there are several proteins that are able to bind/carry LPS. Present in serum are serum albumin, lipoprotein (8,9), soluble CD14 (sCD14), and a 60 kDa glycoprotein with a high affinity for LPS which is called LPS-binding protein (LBP). An LPS-neutralizing protein, which is a 55 kDa bactericidal/permeability-increasing protein (BPI), localizes in a granule fraction of neutrophils (10), but is undetectable in serum.

In the last decade, the pathways by which LPS engages its cellular receptor has been a hot topic in the study of the pathogenesis of infectious disease caused by gram-negative bacteria. It has been shown that sCD14 and LBP play in concert the key role of delivering LPS to target cells. While our understanding of the interaction of LPS with serum proteins or membrane proteins has been greatly advanced, the precise mechanisms remain to be elucidated in different cell types and possibly in different species. It is now known that the differential LPS sensitivity in different cell types results from differential protein receptor expression. An example is that monocytes/macrophages are more sensitive to LPS in respect to the dose used for cell activation than vascular endothelial cells (EC), at least partially because membrane CD14 (mCD14) molecules are expressed on monocytes/macrophages, but are undetected on ECs. In addition, the differential sensitivity to LPS among different species has been recognized, such as the appearance of EC cytotoxicity following LPS challenge in bovine but not human endothelial cells. Therefore, study of the interaction of LPS with
CD14 and LBP in the bovine system appears necessary to demonstrate the similarity and/or difference between human and bovine systems. Furthermore, an objective for this study is to identify the LPS-auxiliary factors in bovine serum, which may benefit biomedical investigators who use bovine serum and bEC as a model.

**LPS, A Ligand For CD14**

LPS is a glycolipid derived from the outer layer of all gram-negative bacteria, consisting of the hydrophilic heteropolysaccharide of the core and O-antigen regions and a hydrophobic domain known as lipid A (11,12).

The outermost part of LPS is the O-specific chain which has a variable length. This constitutes a polymer of repeating hetero-oligosaccharide units (in some cases identical sugar residues) (13), each of which contains up to eight sugar residues. It is able to stimulate the host to generate species-specific IgM antibody that is of little protective effect, but of important diagnostic value in some cases. It has been shown that O-antigen is not required for *in vitro* toxicity of LPS. However, it functions as receptors for bacteriophages, and is involved in the initiation or inhibition of complement activation (14,15), indicating its important role in the pathogenic potential of gram-negative bacteria. In addition, the decreased *in vitro* biological activity due to LPS aggregation and the decreased leukotriene C4 (LTC4) release with increasing completeness of the sugar moiety (15,16) suggest the relatedness of O-antigen
to LPS activity, which is presumably involved in the binding affinity to its receptors.

The core region consists of a hetero-oligosaccharide that can be subdivided into an outer and an inner portion (17). The core is structurally more uniform than the O-chain; structural diversity being found primarily in the outer core. The inner core is characteristically heptose (Hep) and 3-deoxy-D-manno-octulosonic acid (KDO). It has been shown that the minimal LPS structure of growing and multiplying bacteria consists of one KDO residue linked to lipid A, and that optimal lipid A activity is expressed only if at least one KDO residue is covalently attached, indicating that the inner core region can up-regulate lipid A bioactivity.

The lipid A region is embedded in the bacterial cell wall and is responsible for cytotoxic effects. It is present in each LPS molecule (12), and consists of a β1-6 linked diglucosamine backbone containing both ester- and amide-linked long-chain fatty acids as well as pyrophosphate groups. Despite this common architecture, lipid A of different bacterial origin may vary in their fine structure, such as variation in the type of hexosamine, the degree of phosphorylation, the presence of phosphate substitutes, and most notably, the nature, chain length, number, and location of acyl groups (12). With synthesized lipid A or partial structure, it has been shown that the specificity of lipid A binding to the recognition molecule is largely mediated by its hydrophilic backbone while the acyl groups are of central significance in the activation of cells.
CD14, either soluble or membrane-associated forms, serves as a LPS receptor (18), and recruits LPS to its signal transducer on the cellular membrane (19). CD14, unlike LBP which is thought to be an accelerator of the formation of CD14-LPS complexes, is able to form a stable complex with LPS. It is logical and reasonable to hypothesize that the enhanced capacity of cellular activation by LPS in the presence of CD14 is at least due partially to the increased affinity of LPS to its receptor/transducer which results from changed conformation of LPS via the interaction with CD14. The hypothesis is based on many studies indicating that the configuration and size of the LPS aggregates as well as the state of order of the acyl chains should strongly influence the biological activity (15). In addition, EC activation initiated by sCD14-LPS complex can be logically explained by either the improved affinity of LPS to its receptor/transducer or the existence of a membrane protein specific for CD14-LPS complex or both. Thus far, no information is available regarding the conformation of LPS (or lipid A) bound to either LBP or CD14. Such information would be interesting because these protein complexes of LPS mediate the cellular response to LPS (18).

LBP, An Accelerator For Formation Of CD14-LPS Complex

For a long period of time, we have known that serum in in vitro cell culture is required for the production of inflammatory products in response to LPS. Picogram concentrations of LPS in the presence of serum are sufficient
to stimulate target cells such as monocyte/macrophage and vascular endothelial cells, while 100-1000 fold greater LPS concentration is required for cellular activation in the absence of serum or protein factors. The primary understanding of this mechanism was from the discovery of LPS binding protein, a 60-kDa serum glycoprotein that binds to LPS via lipid A (20). LBP has been identified in rats, mice, pigs, and non-human primates by several labs (21), and in serum from cattle by our group (22,23).

LBP is present in normal serum at a range of concentrations of 0.5-10 μg/ml, with levels in acute-phase sera varying widely to above 200 μg/ml (24). LBP is synthesized in hepatocytes and the synthesis is under control of cytokines and steroid hormones (25,26). LBP is a single polypeptide with 452 amino acid residues. The complete primary structure of human and lapin LBP was deduced from cDNA cloning (27), and shares considerable homology with bactericidal/permeability-increasing protein (BPI). Using a truncated form of human LBP, Han et al. demonstrated that the LPS binding activity is contained within the amino-terminal half of LBP, and the CD14 interaction site is found at the carboxyl-terminal half of LBP.

LBP is a major plasma protein responsible for endotoxemic shock (28), which is ascribed to its ability to dramatically enhance macrophage responses by binding LPS (29). LBP has been defined as an accelerator for the formation of sCD14-LPS or LPS-mCD14 complexes, as proposed in several working models for the interaction of LPS with protein factors (9,18,19,30). Juan et
al. (30) utilized recombinant soluble CD14 (rsCD14), either truncated or intact, and confirmed that sCD14 can interact with both LPS and rLBP. LBP increases the formation of sCD14-LPS complex and the cellular adhesion activity induced by LPS in the presence of sCD14. Interestingly, LBP-LPS complex can not be detected in the absence of sCD14, suggesting inability of LBP to form a "stable" complex with LPS. It should be pointed out that the unstable LBP-LPS complex may actually be composed of a continuing and rapid interactive process with LPS, and may be detectable with one method but not with others. Pujin et al. (31,32) demonstrates that LBP dramatically increases the labeling of CD14 by $^{125}$I-LPS with photoaffinity crosslinking methodology, while labeling of the sCD14 is hardly detectable in the absence of LBP. The binding affinity of both LBP and BPI to LPS are quite high, but BPI does not promote the interaction of LPS with CD14. The putative mechanisms include (1) BPI lacks a domain interacting with CD14, or/and (2) the affinity of BPI to LPS is too high to dissociate LPS following binding. High affinity and rapid dissociation are characteristics of LBP binding to LPS, and possibly are necessary for the efficient transfer of LPS. As mentioned above, a mechanistic interpretation of how LBP transfers LPS to CD14 and how LPS changes its configuration to improve its fit into the LPS binding site of CD14 receptor has yet to be explained.
CD14, A LPS Receptor, But Not A Signal Transducer

Cluster of differentiation antigen 14 (CD14) was originally described as a myeloid differentiation marker present on mature peripheral blood monocytes, but not myeloid precursors (33). The gene map and the deduced structure of human CD14 from the sequence of a genomic clone had been described before any function was known (34,35). Its important role in LPS-mediated cell activation was not known until 1990 when Wright et al. discovered that anti-CD14 antibodies blocked the production of tumor necrosis factor α (TNFα) from LPS-stimulated human blood cultures and the binding of LPS-erythrocyte complex to macrophages (36).

In humans and rodents, CD14 has a soluble form (sCD14) in plasma (37,38) and a membrane-bound form (mCD14) on the surface of monocytes and macrophages (34,39-41) and neutrophils (42,43). The human mCD14 receptor is a glyceryl phosphatidylinositol (GPI)-anchored receptor (35,44), and we also have evidence that the bovine homologue of mCD14 is GPI-anchored on bAM (45).

The human CD14 receptor protein was first described as a doublet of approximately 55 kDa in SDS-PAGE (46), and it has recently been confirmed that two bands of CD14 protein appear from supernatants prepared from surface iodinated human monocytes (47). CD14 on neutrophils has similarly been described in two forms, but of 48 kDa and 53 kDa (43). The size difference of the two forms has led to designation as sCD14α (low M,) and
sCD14β (high M,) (48). Both sCD14α and sCD14β are synthesized and released by normal human monocytes and are present in normal plasma. All forms of CD14 may come from a common 50 kDa precursor, which is supported by the presence of a single transcript of 1.5 kb mRNA in human cells. The differences in molecular mass are apparently due to differences in glycosylation, and it is possible that this could be involved in different endocytic-exocytic pathways taken by CD14 in cells (48).

Eight C-terminal amino acids are critical for GPI membrane anchoring and are removed off during this event. sCD14 isolated from urine of a nephrotic patient does not have this C-terminal anchoring signal. Absence of the eight C-terminal amino acids in truncated CD14 transfected CHO does not prevent the release of a sCD14β-like form, but blocks the expression of mCD14 (48).

It is not yet known what functional differences, if any, these forms may have, but different forms of CD14 may differentially regulate induction of cytokine synthesis. In an interesting report from Sundan et al (24), soluble CD14 isolated from the urine (uCD14) of nephrotic patients was shown to contain a potent cytokine inducing activity. Treatment of human astrocytoma cell line U373 with uCD14 resulted in strong secretion of TNF and IL-6. The TNF-inducing activity was also heat labile, in contrast to the cytokine-inducing activity of LPS which was relatively heat resistant. The authors also suggested that CD14 may exist in at least two forms of which one is involved in cytokine induction. Using cDNA truncation, Lee et al. also demonstrated that GPI-
anchored or integral membrane forms of CD14 mediated identical cellular responses to endotoxin (49).

Recent studies indicate that CD14 plays a role in initiating cell activation by bacterial envelope components from gram-positive organisms as well as gram-negative organisms (50,51).

mCD14 receptor expression on cells and the level of sCD14 in human plasma can both be regulated by stimuli. Elevated levels of sCD14 have been found in HIV-1 infected patients (52) and polytraumatized/severely burned patients (37). In vitro experiments show that LPS can induce upregulation of mCD14 on human neutrophils (42), and on the human monocytic cell line Mono Mac 6 (53). LPS can also induce transient increases of mCD14 and associated mRNA in mouse Kupffer cells (54), or can down-regulate mCD14 on human monocytes (47). Cytokines such as interferon-γ (55) and IL-4 (55,56) down-regulate mCD14 expression on human monocytes. In our lab, we have demonstrated that LPS can increase CD14 mRNA expression in bovine alveolar macrophages (45). Because mCD14 is a GPI-anchored protein (35,44), it is susceptible to cleavage and release by agents that activate phosphatidylinositol-specific phospholipase C (PI-PLC). Shedding of CD14 from the cell surface, or receptor-directed "down-regulation," is one possible mechanism for the well-documented desensitization of leukocytes to LPS that occurs with time and repeated administration of LPS. These data collectively demonstrate that levels of mCD14 and sCD14 are dynamic, and are influenced by the presence of
bacterial LPS. These data, plus the knowledge that CD14 is involved in LPS-mediated stimulation of immune cells, indicate that CD14 plays an important role in the response of animals to gram-negative bacteria.

It is now recognized that the CD14 receptor plays an important part in presentation of low concentrations of bacterial LPS. LPS first complexes with LPS-binding protein (LBP) in serum, and the LPS-LBP complex results in a conformational change in LBP, allowing its association with mCD14 molecules on cells (57,58), or sCD14 in plasma (31). The resultant LPS-LBP-CD14 complex triggers activation of certain cells, including macrophages and neutrophils, where the CD14 is a membrane-bound receptor (mCD14). Soluble CD14 in serum or plasma has been demonstrated to be responsible for LPS-mediated activation of endothelial or epithelial cells. In this case, sCD14 can bind LPS directly or with catalysis from LBP, and then deliver LPS to an uncharacterized receptor on the endothelial or epithelial cells (31,46,59-65). In the latter scenario, transfer of LPS to CD14 proceeds more efficiently in the presence of LBP.

Activation of mononuclear phagocytes, neutrophils, and endothelial cells is presumably part of the normal host-defense response of animals to invading gram-negative bacteria, which shed LPS. The association of CD14 with LPS/LBP allows cells of the immune system to respond to very minute concentrations of LPS, which probably lends the ability to detect gram-negative bacteria early in the course of infections. Unfortunately, the association of
CD14 with LPS/LBP also may over-stimulate these cells and result in some of the untoward sequelae associated with these bacterial infections, such as shock and death.

The literature indicates that CD14 on monocytes/macrophages (mCD14) is an essential receptor for recognition of low concentrations of LPS (0.1-10 ng/ml), similar to plasma concentrations that may occur in vivo. High concentrations of LPS (e.g. >100 ng/ml) can directly activate monocytes/macrophages via a controversial pathway, which may involve unknown receptors or receptor-mediated pinocytosis (12,17,66,67). However, these high concentrations do not occur in vivo and are probably not related to the pathogenesis of endotoxin-related diseases. In our lab, we have demonstrated that the presence of bovine serum protein(s) (LBP) and a bovine CD14-like receptor greatly enhance the sensitivity of bovine alveolar macrophages (bAM) to bacterial LPS (23,45,68). We have also demonstrated that binding of LPS to bAM is enhanced in the presence of serum proteins, and that binding can be inhibited by anti-CD14 antibody (22,23,45). In addition, we have shown that low concentrations of LPS require the presence of both bovine LBP and rsCD14 in order to initiate activation of bovine vascular endothelial cells (68). These data and other results from our laboratory suggest that the bovine CD14 receptors exists and plays a role in LPS-associated bovine cellular responses.

It is a controversial issue about the effect of sCD14 on LPS-dependent
activation of cells bearing GPI-anchored CD14. *In vitro* studies revealed that sCD14 has modest inhibitory effects on LPS-stimulated TNF-α production from human monocytes (69), although complete inhibition (with 0.25-0.5 ng/ml of LPS) required addition of huge amounts of sCD14 (50-100 μg/ml). However, a recent study has shown that sCD14 promoted LPS activation of CD14-deficient monocytes and endothelial cells from paroxysmal nocturnal hemoglobinuria (PNH) patient (70). PNH is an acquired disorder in which hematopoietic cells fail to express GPI-anchored proteins, thus mCD14 is deficient while the cell is able to release sCD14 into the blood. Recombinant CD14 enabled complete responses to LPS in both normal and CD14-deficient monocytes. The effective concentration of LPS used in this study was at or above 10 ng/ml. It is possible that at equilibrium, sCD14 forms and dissociates LPS-sCD14 complex dynamically. In the presence of a large concentration of sCD14 and picograms of LPS, e.g. a ratio of greater than 10,000 (10 μg of sCD14 versus <0.5 ng of LPS), sCD14 retains a part of LPS. The portion of LPS is unavailable for cells because it is hypothesized that transfer/dissociation efficiency of LPS is not complete. In contrast to nanogram levels of LPS, LPS transferred from sCD14-LPS complex is sufficient for monocyte activation, because signaling efficiency of LPS is dramatically increased following its interaction with sCD14 as seen in EC activation. It should be noted that the inhibitory effect of rsCD14 on TNF-α production was detected in whole blood, while increased activation of monocytes from PNH patient by rsCD14 was
determined in serum-free medium. The difference can be explained by results of a recent investigation showing that LPS can be transferred to high density lipoprotein (HDL) by sCD14 or a combination of sCD14 and LBP (9). Another assay in this study (70) using PNH monocytes showed that in the absence of serum, anti-CD14 monoclonal antibody 3C10 and My4 inhibited tissue factor production, which was supposed to be due to the small amount of CD14 secreted by these cells during the 6-hour time course of the experiment. Credible data using fluorescein isothiocyanate (FITC)-labeled LPS and anti-CD14 antibodies demonstrated that the addition of sCD14 to 20% serum is able to reduce LPS binding to human monocytes and bovine monocytes at 4°C with the conclusion that endotoxin-neutralizing capacity of soluble CD14 is a highly conserved specific function (71). It is not clear if the reduced binding of LPS imparted by sCD14 is due partially to the failure of dissociation of LPS-sCD14 complex at 4°C. Moreover, the binding performed at 4°C does not represent actual binding of LPS to the cells used in a bioassay at 37°C, and it does not reflect the actual function of sCD14 in either in vitro or in vivo biological reactions at physiologic temperatures. A recent result in our lab indicates that the binding of LPS to bovine macrophages at 37°C is moderately increased by addition of rsCD14. However, rsCD14 in serum-containing medium significantly inhibits LPS binding/uptake by bovine macrophages. It appears that rsCD14 has a dual effect and the neutralizing effect of rsCD14 on LPS binding/uptake is serum-dependent. One part of this study is designed to
further clarify the effect of rsCD14 on LPS-dependent activation of bovine macrophages (tissue factor expression) and LPS binding assays.

As just mentioned, GPI-linked mCD14 on monocytes/macrophages does not have a transmembrane domain to transduce a signal following binding of the receptor ligand, LPS. Therefore it is generally thought that mCD14 must transfer LPS or transmit a signal to an uncharacterized, neighboring transmembrane protein (18, 57), which can then initiate cellular signalling. This "mystery protein" has been hypothesized to be a receptor which associates with a cytoplasmic tyrosine kinase (72, 73). This hypothesis is indirectly supported by a study (74) in which tyrosine kinase inhibitors protected mice from LPS-induced lethal toxicity. This field is still somewhat controversial, however, and another study showed that CD14-mediated translocation of nuclear factor kappa B induced by LPS did not require tyrosine kinase activity (75).

LPS Signal Transducer, An Unknown Transmembrane Component

It is still unclear how the signal from LPS-CD14 interaction is transduced into the intracellular pathway to activate cells. In monocyte/macrophage, mCD14 has been proposed to involve transmembrane signaling via receptor-mediated endocytosis (76) or via interaction with a signal transducer (18). Since ECs or epithelial cells lack mCD14, it is logical to hypothesize that the cellular membrane contains a component which interacts with the sCD14-LPS
complex (18), and possibly serves as a part of signal transducer complex.

In a recent review, Ulevitch and Tobias proposed three models for CD14-associated signal transducing by LPS (18). In model one and two, the conformational change of mCD14 following LPS binding is recognized by a transmembrane protein that itself does not bind LPS. The protein is either specific for the mCD14 GPI-anchor (model one), or part of the LPS receptor complex (model two). In the model three, the transducing molecule directly binds LPS in the absence of CD14, albeit with low affinity. The affinity for LPS is increased in the presence of mCD14. If the model is also applied to ECs/epithelial cells, mCD14 negative cells should contain a protein capable of retaining and interacting with sCD14-LPS complex. The interaction of the protein with sCD14-LPS complex enables sCD14 to facilitate the signaling event. If the protein interacts with sCD14, but not with LPS, another transducing protein that binds LPS may be required. However, the protein interacting with sCD14, if it is transmembranous, may also serve as a signal transducer simultaneously. So far, the protein interacting with sCD14 is not identified, although a report has showed some evidence of the binding of sCD14 to human umbilical vein endothelial cells (HUVEC) (77). We think that model three provides the explanation for CD14-independent signalling, and logically fits both mCD14-bearing and mCD14-lacking cells.

In addition to mCD14, several other membrane-associated proteins which recognize LPS have been reported (67,78). One well-characterized receptor is
the leukocyte integrin CD18, which is capable of binding LPS and participating in the phagocytosis of bacteria. However, CD18 is not essential for mediating cellular response to LPS since cells from CD18-deficient patients were able produce normal levels of TNF-α, IL-1 β, and superoxide in response to LPS. Lei and Morrison identified a potential LPS receptor by using a cross-linking approach. This p73, unlike CD14 whose expression is limited to a small group of target cells (monocyte/macrophage and neutrophils), is widely distributed in splenic B & T cells, neutrophils, monocyte/macrophages, platelets, rat trophoblast, and some cell lines such as 70Z/3, YAC-1, EL-4 J774A.1, L929, COS-7, and P815 cells. Specificity of binding LPS was verified by competitive assay. Functional identification and molecular cloning is required to finally determine its importance and relationship, if any, to the CD14-LPS complex.

If it is the transducing molecule of LPS signaling, and the signal transducer is the same transmembrane protein among a variety of cell types that respond to LPS, its wide distribution is logical since both CD14-bearing and nonbearing cells respond to LPS via an LPS-mCD14-transducer or LPS-sCD14-transducer interaction. The other membrane proteins which have been shown to recognize LPS includes p38, p18, p25, and p65, which are less characterized.

Current Models For Extracellular Pathways Of LPS

The sequence of binding of LPS to the putative signal transducer has been discussed above. A scheme in a recent review by Ulevitch and Tobias
(18) illustrates how LPS interacts with myeloid and nonmyeloid cells via an LBP/CD14-dependent mechanism. In the proposed pathways, a role for mCD14 has been defined in LPS activation of myeloid cells, while sCD14 has been shown to participate in activation of nonmyeloid cell types such as endothelial or epithelial cells that normally do not express mCD14. Briefly, LPS binds LBP to form LPS-LBP complexes which interact with sCD14 or mCD14 to accelerate the formation of LPS-CD14 complexes. LPS-sCD14 complexes trigger nonmyeloid cell activation, while LPS-mCD14 triggers myeloid cell activation. Interaction of the complexes with an unknown cellular receptor or signal transducer appears essential for both myeloid and nonmyeloid cell activation. It is also proposed that LPS forms a ternary complex with LBP and mCD14 (19). By using monoclonal MAbs for LBP and mCD14, internalization of the LPS-LBP complex and mCD14-signal transducer pathway can be separated in these designed experiments. Evidence includes that anti-LBP antibody 18G4 inhibits LBP binding to mCD14 without blocking signal transduction or LPS transfer to soluble CD14, while anti-CD14 antibody 18E12 inhibits signal transduction without affecting LPS binding and uptake. A conclusion deduced from the experiments is that while LPS signal transduction and LPS clearance utilize both LBP and mCD14, the pathways bifurcate after LPS binding to mCD14.

We now know that LPS-sCD14 formation plays a central role in nonmyeloid cell activation. A question is whether or not LPS is able to directly
bind mCD14 to trigger cellular activation. Because the difference between sCD14 and mCD14 is only a 2 kDa GPI linker, LPS is expected to directly bind mCD14, bypassing the requirement of LBP. This interaction is quite weak. A study with the HTP-1 cell line (human myelomonocytic leukemia) that expresses a high number of mCD14 following vitamin D3 treatment has shown that anti-CD14 MAb My4 inhibited LPS-induced interleukin 8 (IL-8) production in the absence of LBP (79). Inhibition by MY4 on LPS-induced membrane IgM expression (without LBP) on the human CD14-transfected 70Z/3 cell line is seen from a published study (80). However, such a result has not been seen from any "normal", nontransformed cells. It appears that the affinity of mCD14 for free LPS is much weaker than that of sCD14. Hypothetically, the decreased affinity of mCD14 for free LPS could be due to a conformationally imperfect match or/and the interference from glycocalyx on the cell surface.

Another controversial issue as mentioned above is whether sCD14 increases LPS-dependent activation of mCD14-bearing cells. From available information (69,70), it appears that entirely different conclusions can be drawn when employing different experimental conditions, especially with respect to LPS concentration and existence of serum in assays. This phenomenon may be explained with our "three steps-efficiency" model (see Figure 5-8 on page 142), which is derived from both our data and other publications. In the presence of picogram concentrations of LPS, LBP is required to interact with sCD14 or mCD14 for target cell activation. Excessive sCD14 may sequester
some LPS since the dissociation of LPS from sCD14 may not be complete. At slightly higher nanogram concentrations of LPS, sCD14 may transfer LPS onto cellular surface for cell activation. LPS transferred by sCD14 is of high affinity for membrane receptors, regardless of whether the receptor is an LPS signal transducer or mCD14. At high concentrations of LPS, LPS is able to directly bind to a putative signal transducer in sufficient quantity to trigger cell activation, e.g. in a serum-free response with inhibition by anti-CD14 MAb. It appears that the routes ("steps") taken by LPS determine the efficiency of cellular stimulation. LPS transfer and binding via sequential interactions of LPS-LBP, LPS-CD14, and finally LPS-transducer (i.e. "three steps"), has the highest efficiency of stimulation on the target cells. In the absence of LBP (two steps), the efficiency is either moderate (LPS-sCD14 for EC and monocytes) or low (LPS-mCD14 for monocytes). The lowest efficiency is one step, namely the direct binding of LPS to a putative signal transducer. Since there are no precise mechanisms available for explaining the relationship between the interactions of these protein factors and the efficiency of LPS, recruitment of LPS to signal transducers by these protein factors and configurational change of LPS following interactions are presumably responsible for the differential efficiency among different steps. Some supportive evidence for this model has been obtained from one part of this study using LPS-dependent tissue factor expression and an LPS binding assay.
Significance Of Studying Bovine CD14 Receptors

The molecular basis of LPS-mediated morbidity and mortality is largely a result of work accomplished by investigators using human, murine, and rabbit systems.

As stated previously, the interactions of LBP and CD14 with LPS may function to enhance host defense against gram-negative bacteria by enabling detection of small quantities of LPS early in the course of infection. As such, CD14 probably plays an important role in the pathogenesis of gram-negative bacterial diseases, including those in cattle. Bovine calves are very sensitive to bacterial LPS, exhibit endotoxemic signs and correlative clinical laboratory data at LPS concentrations of only 0.5 μg/Kg body weight (81), and are comparatively much more sensitive than some other common domestic and laboratory species such as mice, rats, and rabbits (82,83). Another well-known example is that LPS mediates cytotoxicity to bovine EC, but not to human EC. The intrinsic difference in response to LPS stimulation between humans and cattle is one of the driving forces for us to study bovine CD14. Characterization and study of bovine CD14 and the LPS-LBP-CD14 interaction should provide new insights into how gram-negative bacteria and bacterial LPS induce changes that lead to morbidity and mortality in cattle.

To our knowledge, our lab is unique in study of bovine LBP and bCD14 molecules, although there are some publications involving functional assays using anti-CD14 antibodies and bovine EC (46,63). Unfortunately, a misleading
conclusion was drawn from the latter studies. Existence of bovine CD14 was reported as doubtful, since anti-human CD14 MAbs did not inhibit bovine serum-dependent damage of bovine EC by LPS. These investigators did not examine the effect of anti-CD14 antibodies with concentrations of bovine serum <5%. It is well known that biological dose-response is sometimes critical, especially when a new experimental system is used. We have carefully determined both antibody-dose and serum-dose responses, and conclude that bovine serum contains sCD14 receptors specifically recognized by some anti-human CD14 MAbs. At >5% bovine serum, the anti-CD14 antibody is effective for inhibition of human, but not bovine serum-dependent EC response to LPS. In addition to the unknown concentration of sCD14 in bovine serum, an additional explanatory factor is that the affinity of anti-human CD14 MAbs to bovine CD14 may be weaker than to human CD14. Clarification of the existence of bovine CD14 and the interaction of these receptor proteins with LPS is not only a part of study of inflammation in veterinary medicine, but also an important issue in general biomedical research because the bovine serum and EC are widely used in a variety of fundamental and clinical investigations. We have also made progress on this front in our lab using bovine cells and bovine proteins. Bovine CD14 has been confirmed by functional assay, northern blot, western blot, hybrid-selected translation, and immunoprecipitation (23,45,68,84). Our results also indicate that the extracellular pathway of LPS transfer in the bovine system is very similar to
that reported in the human system. This conclusion could not have been made without the results of this study. In addition, by using bovine macrophages and recombinant sCD14, we have demonstrated enhanced binding of LPS to bAM in the presence of sCD14. This observation may expand current understanding of the interaction of LPS with CD14, and resultant LPS stimulation of both myeloid and nonmyeloid cells.

References


Transfection of CD14 into 70Z/3 cells dramatically enhances the sensitivity to complexes of lipopolysaccharide (LPS) and LPS binding protein. *J. Exp. Med.* 175:1697-1705.


PART 2

CD14 AND TISSUE FACTOR EXPRESSION BY BACTERIAL LIPOPOLYSACCHARIDE-STIMULATED BOVINE ALVEOLAR MACROPHAGES IN VITRO

Abstract

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Figures 2-1 to 2-6
Abstract

The membrane-associated CD14 receptor (mCD14) is a monocyte/macrophage differentiation antigen and has been demonstrated to serve as a receptor for bacterial lipopolysaccharide (LPS, endotoxin). Binding of LPS to mCD14 has been shown to be associated with LPS-induced macrophage, monocyte, and neutrophil activation in humans. In these studies, we describe the presence and function of a mCD14-like receptor on bovine alveolar macrophages (bAM). Immunofluorescence technique and flow cytometric analysis indicated binding of anti-human CD14 monoclonal antibodies (MAb) My4, 3C10, and 60bd to bAM. Binding of anti-CD14 MAb (3C10, MY4) was reduced over 20% by pretreatment of bAM with phosphatidylinositol-specific phospholipase C (0.5-1.0 U/ml), indicating that bovine mCD14 is a glycosyl phosphatidylinositol-anchored protein. In addition, pretreatment of bAM with anti-CD14 MAb decreased binding of $^{125}$I-labeled LPS to macrophages, suggesting that bovine mCD14 serves as a receptor for LPS. A cDNA probe based on the human sequence for CD14 was used in Northern blot analysis, and hybridization to human monocyte CD14 yielded the expected 1.5 kb band. Hybridization to bovine mRNA yielded a 1.5 kb band plus an unexpected 3.1 kb band. Constitutive expression of bovine CD14 mRNA was observed, and the expression level was modestly elevated in bAM stimulated for 24 hours with LPS (1 ng/ml) in the presence of bovine serum. Function and activation of bAM was assessed by quantitation of tissue factor (TF) expression.
on the cells using an activated factor X-related chromogenic assay and S-2222 substrate. LPS (1 ng/ml)-mediated upregulation of TF expression on bAM was dependent on the presence of bovine serum components, and TF expression was inhibited by anti-CD14 MAb. In addition, TF mRNA levels in LPS-stimulated bAM were decreased by pretreatment of cells with anti-CD14 MAb (MAB 60bd, 10 μg/ml).

Introduction

Bacterial LPS is a potent toxin released from gram-negative bacterial cells walls, and it is notorious for initiation of inflammation and medical dilemmas (28). Cells of macrophage lineage are exquisitely sensitive to the presence of LPS, and when activated by the presence of this bacterial product may orchestrate a multitude of proinflammatory processes (8,42). In the lung, alveolar macrophages play prominent regulatory roles in host defense, but in some circumstances the macrophages may initiate edema formation, excessive leukocyte infiltration, and fibrin deposition in alveolar air spaces in response to the presence of gram-negative bacteria and LPS (46,47).

Factors involved in the interaction of LPS with myelomonocytic cells have recently been elucidated. Lipopolysaccharide-binding protein (LBP) is a 58-60 kD glycoprotein present in the sera of humans (32), rabbits (40), and mice (13), and is an acute-phase protein which increases in concentration in response to inflammatory stimuli. LBP in serum binds LPS with high affinity.
(32, 31, 44, 49), and the resultant LBP-LPS complex binds to mCD14 receptors on the surface of monocytes, macrophages, and neutrophils. The 53-55kD mCD14 receptor molecule has been identified on cells of human and murine origin, and this receptor possesses a glycosyl phosphatidylinositol anchor (16, 34). Other LPS receptors have also been described (22). A soluble form of CD14 is also normally present in human plasma (2), lacks the lipid tail, and has a slightly lower molecular weight (48-50 kD) compared to its membrane-bound cousin. LBP has been found to shuttle LPS to the soluble form of CD14 (14), utilizing the amino-terminal half of LBP for LPS binding, and the carboxyl-terminal half for interactions with CD14 (15). Interaction of LBP and mCD14 with LPS amplifies the response of macrophages to LPS, and results in generation of proinflammatory cytokines such as TNFα and IL-6 (10, 27, 32, 50). Formation of proinflammatory and immunomodulatory cytokines as a result of exposure to LPS may be beneficial to the host, but excessive cytokine synthesis may be detrimental.

The lung is a target for gram-negative bacterial infections in many species, including cattle, and interactions of alveolar macrophages with LPS are important in determining the outcome of these infections (9, 47). In research laboratories, many investigators utilize bovine sera or cells for in vitro studies, but published work has not clarified the factors involved in interactions of bovine-origin cells or fluids with LPS. A limited amount of bovine-related data exists, including reports that uncharacterized bovine serum factors bind LPS
(19) and enhance association of LPS with peripheral-blood leukocytes (5). Bovine serum factors also promote activation of bovine macrophages in the presence of LPS (50). Our objective in the current study was to further characterize the factors involved in LPS-mediated activation of bovine alveolar macrophages. Results suggest the presence of mCD14 on bAM, and indicate that bAM are dependent on a mCD14-like receptor and LBP-like serum factors for LPS-mediated cellular activation and tissue factor expression. The findings suggest similar function of bovine mCD14 to that reported for human mCD14.

**Materials and Methods**

**Bovine Alveolar Macrophages**

Bovine alveolar macrophages were collected from 1-3 month-old Holstein-Frisian calves by volume-controlled bronchopulmonary lavage as has been described (21). Lavage fluid with cells was filtered through sterile gauze, cells were pelleted by centrifugation, and were resuspended and washed twice in sterile, pyrogen-free buffer before final resuspension in Dulbecco’s Modified Eagle’s Media (DMEM). The percentage of macrophages determined by Wright-Giemsa stained cytocentrifuge preparation was greater than 90%, and viability determined by trypan blue dye exclusion was also greater than 90%. Protocol for collection of alveolar macrophages was approved by an animal care committee at this institution.
Tissue Factor Assay

Tissue factor expression on the macrophages was quantitated with a colorimetric assay (35) based on digestion of substrate S-2222 by activated factor X. The assays were performed as previously described by our laboratory (50). Treatment variables included macrophages exposed to various concentrations of LPS (E. coli O55:B5) alone or in combination with quantities of fetal bovine serum (FBS), or a bovine serum chromatography fraction (described below). In some assays monoclonal antibodies or isotype-matched MAb of irrelevant specificity (control MAb) were added 20 minutes before addition of the LPS+ serum. We found the colorimetric assay for tissue factor to be very consistent with that of the modified prothrombin assay (6).

Bovine Serum/Serum Chromatography

Pooled bovine serum collected from normal, healthy cows was fractionated using Bio-Rex 70 chromatography media as previously described for rabbit serum (40) and used with bovine serum (50). Fifty ml of Bio-Rex resin was equilibrated with 41 mM NaCl in 50 mM phosphate buffer, pH 7.3, containing 2 mM EDTA (phosphate-EDTA). Two hundred ml of bovine serum containing 5 mM EDTA, was run over the column at 60 ml/hour. The column was then washed with column equilibration buffer until the absorbance (280 nm) of the eluate was <0.2 absorbance units (AU). Washing was continued with 220 mM NaCl in phosphate-EDTA until the absorbance was <0.2,
followed by a linear gradient formed from 60 ml each of 220 mM and 500 mM NaCl in phosphate-EDTA. Finally, the column was washed with 1 M NaCl in phosphate-EDTA. Pools of fractions were dialyzed vs 5 mM Hepes, pH 7.3, and concentrated approximately 10-fold using YM10 membranes in an Amicon ultrafiltration cell. The bovine serum chromatography fraction used in our current work corresponds to the column eluate obtained when washing with 220 mM NaCl.

**Immunofluorescence Assay**

bAM were first preincubated with murine IgG (20-100 µg/ml) in phosphate-buffered saline (PBS, Ph 7.3) for 30 minutes to block nonspecific binding sites, followed by addition of murine anti-human CD14 antibodies (10 µg/ml) or another non-CD14 antibody known to bind to bovine macrophages. The latter was MAb 60.3, a mouse-anti-human IgG\textsubscript{a} that bound avidly to bAM in our own studies and which recognizes the CD18 cell surface glycoprotein complex (3). The specificity of MAb 60.3 for bovine neutrophils has been verified previously (4), and the CD18 complex on bovine leukocytes documented (33). After incubation at 37°C for 30 minutes, cells were washed twice with PBS, then incubated for 10 minutes with 5% goat serum to block nonspecific binding sites. Fluorescein isothiocyanate (FITC)-labeled goat antimouse IgG (20 µg/ml) was then added for a 30 minute incubation period. bAM were then washed twice and fixed with 0.5% paraformaldehyde. Fluorescence
analysis was accomplished utilizing a Becton-Dickinson FACScan with analysis
gate set to collect and quantitate the FITC-antibody associated with bAM. 10,000 cells were counted per treatment and data expressed as relative mean fluorescence intensities. In the experiments using PI-PLC, bAM were preincubated with PI-PLC (0.01-1.0 units/ml) for 1 hour at 37°C, then washed twice with HBSS, followed by the antibody labeling procedure just described.

Binding of $^{125}$I-labeled LPS to bAM

LPS from E.coli 0111:B4 was sonicated, then labeled by first coupling to SASD as described (48). The LPS-ASD complex was radiolabeled with Na$^{125}$I using chloramine T (43,48). The product ($^{125}$I-ASD-LPS) regularly had a specific activity of 6 x 10$^6$ cpm/mg LPS. Bovine alveolar macrophages were pre-incubated with anti-CD14 antibody 60bd (20 μg/ml) or an isotype-matched MAb of irrelevant specificity for 30 min, then incubated with $^{125}$I-ASD-LPS with/without bovine serum for 1 hour in the dark at 37°C, 5% CO₂, and ambient humidity. Bound ligand ($^{125}$I-ASD-LPS) was then subjected to photolysis for 10 minutes at room temperature using short-wave UV irradiation (Hoefer Scientific Instruments, CA., UVC 1000 cross-linker, maximal emission at 254 nm). $^{125}$I-ASD-LPS was linked (photolysis) to molecules on the macrophages with which LPS became associated. Cells were subsequently fixed with 0.5% paraformaldehyde for 10 minutes, then pelleted by centrifugation and washed three times with PBS to remove unbound radioactivity. Macrophages were
counted for gamma emission as an indication of bound LPS using standard methodology.

**Northern Blot Analysis**

Total cellular RNA was purified as described previously using the standard guanidine thiocyanate/CsCl density centrifugation procedure (24). RNA pellets were redissolved in diethylpyrocarbonate-treated, double-distilled water and precipitated by centrifugation from a 70% ethanol/0.3 M NaAc (pH 5.2) solution. Pellets were rinsed with 70% ethanol, dried in a vacuum desiccator, and dissolved in 10 mM sodium phosphate buffer (pH 7.0). RNA concentration was determined by measurement of UV absorbance at 260 nm. RNA was fractionated by electrophoresis in a 1% agarose gel, transferred by capillary action (39) to Biodyne transfer membranes (Pall Biosupport, East Hills, NY), and immobilized by UV irradiation with a UV crosslinker (Hoefer, San Francisco). Blots were pre-hybridized for 3 hours at 42°C (50% formamide/5X SSC, pH 7.0/50mM NaPO₄, pH 7.0/250 μg/ml ss DNA/5X Denhart’s solution/0.1% SDS) and hybridized overnight at 42°C with ³²P-labeled probes (Amersham Multiprime DNA labeling system, Arlington heights, IL) in a similar mixture with dextran sulfate added to a final concentration of 5% (39). The hybridized blot was washed 2 X 10 minutes in 2X SSC/0.1% SDS at room temperature, followed by three 20 minute washes in 0.1X SSC/0.1% SDS at 55°C. Blots were subsequently autoradiographed with preflashed Kodak X-
Omat XAR-5 film and an intensifying screen at -70°C for an appropriate period (1-3 days). Uniformity of RNA loading on the gels was verified by subsequent hybridization to the appropriate α-tubulin gene probe (23). Experiments were repeated with fresh cells to confirm results.

**Reagents and Supplies**

Dulbecco’s Modified Eagle’s Medium was purchased from Whittaker MA Bioproducts, Walkersville, MD, and FBS was obtained from Hy Clone Laboratories, Inc. Logan, UT. The FBS had total protein of 3.2 grams per dL, and contained 0.06 endotoxin units per milliliter as determined by the manufacturer. *Escherichia coli* LPS, serotypes O55:B5 and O111:B4, were purchased from Sigma, St. Louis, MO, and List Biological Laboratories, Inc. Campbell, CA. Phosphatidylinositol-specific phospholipase C was purchased from Boehringer Mannheim, Indianapolis, IN. Sulfosuccinimidyl-2-(p-azidosalicylamido)1,3′-dithiopropionate (SASD) and chloramine T were from Pierce Chemicals, Rockford, IL. The ^125^I-Na was obtained from ICN, Costa Mesa, CA. BioRex-70 resin was from Bio-Rad Laboratories, Richmond, CA, and the YM10 filtration membranes were obtained from Amicon Corp., Danvers, MA. Chromogenic substrate B2-Ile-Glu-Arg-P-nitroanilide (S-2222) was from Kabi Pharmaceuticals, Franklin, OH, and a coagulation factor concentrate (Proplex T) was obtained from Travenol Labs, Inc., Glendale, CA. FITC-labeled goat-anti-mouse antibody and control antibodies were obtained from Organon.
Teknika Corp., Malvern, PA. The two control isotype-matched MAb used in these experiments were mouse IgG1 (Kappa), derived from MOPC 21, a mineral oil-induced plasmacytoma, and mouse IgG2b (Kappa), derived from MOPC 195, also a mineral oil-induced plasmacytoma. Neither of these have any known hapten or antigen binding activity. The murine anti-human CD14 MAb 3C10 (IgG1) was a generous gift from Dr. Samuel D. Wright, Rockefeller University, New York. Murine anti-human CD14 MAb 60bd (IgG1, provided as ascites) was a kind gift from Dr. Robert F. Todd III, Division of Hematology and Oncology, University of Michigan Medical School, Ann Arbor, Michigan. MAb 60bd was purified with an ImmunoPure (G) IgG Purification Kit (Pierce, Rockford, IL) and stored without preservative at -70°C until use. My4, another murine anti-human CD14 monoclonal antibody (IgG2b), was purchased from Coulter Immunology, Hialeah, FL. MAb 60.3 (anti-CD18) was a kind gift of Dr. John Harlan, University of Washington, Seattle, WA. The cDNA probe for human CD14 (34) was generously provided by Dr. Brian Seed, Department of Molecular Biology, Massachusetts General Hospital, Boston, MA. The bovine tissue factor cDNA probe (36) was a kind gift from Dr. Sadaaki Iwanaga, Kyushu University, Fukuoka, Japan. Salmon sperm DNA and α-P<sup>32</sup>CTP were from DuPont/NEN, Boston, MA. Other reagents for the Northern blots were purchased from GIBCO/BRL, NY.
Results

Immunofluorescence Flow Cytometric Analysis

Anti-human CD14 monoclonal antibodies were tested for cross reactivity with bAM, and three monoclonal antibodies (3C10, 60bd, and My4) were found to bind based upon fluorescence flow cytometric analysis (data not shown). Membrane-associated CD14 is a glycosyl-phosphatidyl-inositol-anchored receptor on human cells (16,34), and we therefore tested the effect of PI-PLC pretreatment of bAM prior to antibody labeling. PI-PLC pretreatment reduced the relative fluorescence intensity of antibody-labeled cells, indicating that binding of 3C10 MAb to bAM was reduced in a dose-dependent manner (Figure 2-1A). Whereas pretreatment of macrophages with PI-PLC decreased binding of the two anti-CD14 MAbs tested (3C10, MY4), PI-PLC treatment did not decrease binding of MAb 60.3 which recognizes the CD18 leukocyte integrin (Figure 2-1B). CD18 is not a phospholipid-anchored receptor. Experiments were repeated three times; results from one experiment are shown in figures 2-1A and 2-1B.

Anti-CD14 MAb Decreases Binding of \(^{125}\text{I}\)-labeled LPS to bAM

Previous reports indicate that binding of LPS to human macrophages is dependent upon mCD14 and soluble serum factors such as lipopolysaccharide-binding protein. In order to investigate the requirements for binding of LPS to bAM, binding of \(^{125}\text{I}\)-labeled LPS to bAM was quantitated in the presence of
serum and anti-CD14 MAb. Association of LPS with bAM was dependent on the concentration of LPS (Figure 2-2A). At all concentrations of LPS, the amount of cell-bound LPS was markedly increased by the presence of bovine serum (Figure 2-2A). We employed one of the anti-CD14 MAb that had already been shown to bind to bAM in these assays (MAb 60bd, 20 μg/ml), and found that the anti-CD14 antibody decreased association of LPS with macrophages (Figure 2-2B). Anti-CD14 MAb 60bd diminished total binding of 125I-labeled LPS to bAM by 43% in the presence of 0.5% FBS, by 24% with 1.0% FBS, and isotype-matched MAb of irrelevant specificity did not interfere with LPS binding. Results shown in Figure 2-2A and 2-2B are means from duplicate samples from one of two similar experiments.

**Northern Blot Analysis of bAM CD14 mRNA**

Bovine alveolar macrophages were stimulated with LPS (1 ng/ml) with 0.2% FBS for periods of time up to 24 hours, and a CD14 cDNA probe based on the human sequence was hybridized with RNA purified from the bAM. RNA purified from human monocytes was included as a control in the experiments. Conditions of stringency for post-hybridization washing were 0.1 X SSC with 0.1% SDS buffer for three washes at a water bath temperature of 55°C. In Figure 2-3, a band with the expected size of 1.5 kb was present in human monocyte RNA (left), and in RNA from bAM (right). Unexpectedly, an additional 3.1 kb band appeared associated with the bovine cells. The level of
CD14 mRNA expression appeared greatest in bAM at 24 hours post stimulation with LPS.

**Anti-CD14 MAbs Inhibit Tissue Factor Expression on bAM**

The chromogenic assay based on activation of factor X was used to assess tissue factor expression as a measure of macrophage activation. Our results utilizing the chromatographically-derived bovine serum fraction were similar to those previously reported from our laboratory (50). Similar to these results, current experiments utilizing FBS indicated that components in fetal bovine serum markedly enhanced the stimulatory effect of LPS (1 ng/ml), but LPS alone had little stimulatory effect (Figure 2-4). Stimulation of macrophages was inhibitable by treatment of bAM with anti-CD14 MAb (60bd, 20 µg/ml), but not by an isotype-matched MAb of irrelevant specificity (20 µg/ml). A similar inhibitory effect was also observed when employing another anti-CD14 MAb, MY4. Anti-CD14 monoclonal antibody reduced LPS-serum-dependent tissue factor expression over a range of FBS concentrations from 0.1% to 1% (vol/vol). The inhibitory effect of anti-CD14 MAb was greatest at low serum concentrations. In the presence of 0.1% FBS, tissue factor expression in LPS-stimulated macrophages was reduced over 90% by anti-CD14 MAb (60bca, 20 µg/ml). The inhibitory effect was slightly greater than 20% with 1% FBS, and the antibody was not able to block LPS-stimulated tissue factor expression with 5% FBS. Loss of antibody-related effect at a concentration of 5% serum may
occur, in part, because sCD14 in the serum binds the MAb and prevents access of the antibody to the membrane-bound CD14 receptors on macrophages. It is also plausible that the anti-human CD14 monoclonal antibody has less affinity and blocking effect with mCD14 of a different species, i.e. bovine. Nevertheless, the data support the existence and function of bovine mCD14 on alveolar macrophages.

**Regulation of Tissue Factor mRNA Expression by bAM**

For Figure 2-5, alveolar macrophages were stimulated for various time periods with LPS (1 ng/ml) plus either A) FBS (0.2%, 64 μg/ml protein) or B) a bovine serum-derived chromatography fraction (0.5 μg/ml protein) and RNA was purified. Northern blot analysis revealed that tissue factor mRNA expression was strongest from 4 to 8 hours poststimulation, and was diminished after 24 hours. Similar experiments were conducted using a single time point of 5 hours stimulation and treatment variables including the presence/absence of LPS (1 ng/ml), FBS (0.2%), bovine serum-derived chromatography fraction (0.5 μg/ml), anti-CD14 MAb 60bd (10 μg/ml), and an isotype-matched MAb of irrelevant specificity (Figure 2-6). bAM alone, bAM in 0.2% FBS alone, or bAM treated with 1 ng/ml LPS in the absence of FBS showed absence or low levels of TF mRNA transcripts (A, left panel). When treated with 1 ng/ml LPS in the presence of 0.2% FBS, the TF mRNA level was remarkably increased with the expected band appearing at 2.3 kb. Tissue
factor mRNA levels were lower when cells were treated with anti-CD14 MAb 60bd (10 μg/ml), but not by treatment with the isotype-matched MAb of irrelevant specificity. Similar results were obtained when employing the bovine serum chromatography fraction (B, right panel), except that the serum protein fraction alone induced no signal, whereas FBS alone (left panel) had a slight stimulatory effect. These results indicated that a CD14-like receptor on bAM participated in LPS + serum protein-induced tissue factor mRNA induction.

Discussion

Bacterial lipopolysaccharide in the blood of mammals may bind to several different serum components, but binding to LBP and soluble CD14 is notable due to its high avidity (14,32,44). Binding of LPS to LBP accelerates subsequent delivery of LPS to sCD14 (14), and LBP-LPS complexes also interact with mCD14 on monocytes, macrophages, and neutrophils (17,32,42,45). This serves to enhance the sensitivity of responsive cells to the presence of LPS (10,12,18,25,32), but over-responsiveness may also precipitate immune-mediated septic shock (11,28-30). We have focused our current work on alveolar macrophages because these cells respond to bacterial LPS in combination with soluble proteins of either pulmonary or vascular origin, and investigators have demonstrated that LBP and sCD14 are present in some cases of acute pulmonary inflammation (25,26).

Investigators utilizing human monocytes (16) or CD14-transfected cell
lines (34) have demonstrated that treatment of cells with PI-PLC reduces
binding of anti-CD14 MAbs. This is attributable to the fact that the glycosyl-
phosphatidylinositol anchor of mCD14 is susceptible to cleavage by specific
phospholipase C, and we have similarly shown that binding of anti-human
CD14 MAbs to bovine alveolar macrophages is reduced by pretreatment of
bAM with PI-PLC. This suggests that the antigen on bovine cells recognized
by the antibodies has a phospholipid anchor, as is the case for mCD14.
mCD14 is known to serve as a receptor for LPS on human and rabbit
macrophages (25,32). We have found that radiolabeled LPS binds to bAM, and
that the presence of serum enhances binding of LPS to the cells. In addition,
anti-CD14 MAb decreased binding of LPS to bAM in the presence of serum.
Our findings with bovine cells are consistent with the mechanism of interaction
of LPS with monocytes and macrophages of other species, which dictates that
LPS binds to LBP in serum and enhances binding to mCD14 on the surface of
cells (42,44). Our findings with pulmonary-derived bovine macrophages are
also in concert with those using rabbit alveolar macrophages, where
investigators found that rabbit and human alveolar macrophages respond to LPS
and LBP in a mCD14-dependent manner (25).

Our studies indicate that mRNA encoding the mCD14 receptor is present
in bAM, which is consistent with our findings using anti-CD14 MAbs which
bind to these cells. The bovine mRNA appeared as a 1.5 kb band, which was
similar in size to the human monocyte CD14 mRNA used as controls, and to
that reported for human CD14 in the literature (34). The additional 3.1 kb band on the blots from bovine cells was unexpected, and its significance is unknown. We also found that the mRNA transcript level for CD14 in bovine macrophages was increased after stimulation with LPS for 24 hours. mCD14 receptor transcripts and surface receptor expression have been reported to be increased in the human monocytic Mono Mac 6 cell line by stimulation with LPS or by PGE$_2$ expression, but transiently decreased (51) or increased (38) by phorbol ester. Low concentrations of LPS (0.1 to 1.0 ng/ml) increased mCD14 receptor expression on human monocytes, but expression as measured by flow cytometry was variably decreased with high LPS concentrations (100 ng/ml) (20). Increased levels of mRNA transcripts for mCD14 are related to both increased surface expression of mCD14 on cells and increased shedding of CD14 into the extracellular fluid (51).

Our data also indicated that activation of bAM by LPS was enhanced by the presence of bovine serum or soluble bovine serum factors, and that activation was inhibitable by anti-CD14 MAbs. Tissue factor mRNA levels in bAM were elevated by two hours of stimulation with LPS, peaked at six to eight hours, and slightly decreased by 24 hours. Northern blot analysis of tissue factor mRNA levels from bAM was consistent with the findings in cell assays measuring tissue factor expression, and indicated that LPS-induced tissue factor synthesis is dependent upon mCD14. Tissue factor is a transmembrane protein and functions as a major cellular initiator of the clotting
Increased expression of tissue factor is related to the pathogenesis of gram-negative bacterial diseases, and is a marker of LPS-mediated macrophage activation (7,37). Our finding that LPS-mediated activation of bAM is dependent on CD14 is comparable to CD14-dependent stimulation of human and rabbit macrophages by LPS and LBP (10,25,49).

In summary, our finding indicate the existence of mCD14 on bovine alveolar macrophages. Results indicate that bAM are dependent on a mCD14-like receptor and soluble serum factors for LPS-mediated cellular activation and TF expression, elucidating likely pathways of pulmonary inflammation in bovine pneumonia due to gram-negative bacteria. The data also indicate comparable function of the proposed bovine mCD14 receptor to that reported for human mCD14, suggesting conservation of mechanisms of macrophage responsiveness to bacterial LPS.

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References


Figure 2-1. Binding of anti-CD14 monoclonal antibodies to bovine alveolar macrophage is decreased by PI-PLC pre-treatment. (A) Macrophages were preincubated with several concentrations of PI-PLC (0.1 - 1.0 U/ml) followed by indirect immunofluorescence flow cytometry using MAb 3C10. (B) Macrophages were exposed to a single concentration of PI-PLC (1.0 U/ml), then similarly labeled with anti-CD14 MAbs (3C10, MY4; 10 μg/ml) or anti-CD18 MAb (60.3; 10 μg/ml) and analyzed by indirect immunofluorescence flow cytometry. Results are expressed as percent reduction of antibody binding. Results from one of three similar experiments are shown.
Figure 2-2. Binding of $^{125}$I-labeled LPS to Macrophages. (A) Association of several concentrations of $^{125}$I-labeled LPS with bAM was quantitated in the presence/absence of normal pooled bovine serum (5%), in order to determine whether soluble serum components influence binding of LPS to cells. Results are expressed as LPS-associated gamma counts (CPM) from macrophages. (B) The effect of anti-CD14 MAb (60bd, 20 μg/ml) or an isotype-matched MAb of irrelevant specificity on binding of $^{125}$I-LPS (100 ng/ml) to macrophages in the presence of soluble serum components is shown. Results are expressed as percent reduction of LPS bound to antibody-treated macrophages, as determined from LPS-associated gamma counts. Results shown in figure 2A and 2B are means ± S.D. from duplicate samples from one of two similar experiments.
Figure 2-3. Northern Blot Analysis of Bovine CD14 mRNA from bAM. A band of the expected 1.5 kb size was present in RNA from human monocytes (left lane), while RNA from bAM showed the expected 1.5 kb band and an additional 3.1 kb band. Some bAM were stimulated up to 24 hours with LPS (1 ng/ml) and FBS (0.2%) before RNA was purified. Results were similar for two blots derived from separate isolates of macrophages.
Figure 2-4. Anti-CD14 MAb inhibited LPS-dependent tissue factor expression by bovine alveolar macrophages. The assay was used to detect activation of macrophages, and anti-CD14 monoclonal antibody was employed to demonstrate the role of mCD14 in cellular activation. (A) Tissue factor expression/macrophage activation is proportional to the increase in optical density, and is dependent upon the presence of fetal bovine serum. Data are means ± S.D., n = 4. (B) The data are calculated to reflect the inhibitory effect of the anti-CD14 monoclonal antibody (60bca, 20 μg/ml), as compared to the cells receiving a control antibody of irrelevant specificity (IgG₁, 20 μg/ml); means of 4 determinations.
Figure 2-5. Kinetics of expression of TF mRNA by Macrophages. Alveolar macrophages were stimulated for time periods up to 24 hours with LPS (1 ng/ml) plus either A) FBS (0.2%, 64 µg/ml protein) or B) a bovine serum-derived chromatography fraction (0.5 µg/ml protein). Purified RNA was then probed using bovine tissue factor cDNA. Experiments were repeated twice to confirm results.
Figure 2-6. Regulation of Tissue Factor mRNA Expression by Macrophages. Experiments were conducted using macrophage treatment variables including the presence/absence of LPS (1 ng/ml), FBS (A, left panel, 0.2%), bovine serum-derived chromatography fraction (B, right panel, 0.5 μg/ml protein), anti-CD14 MAb 60bd (10 μg/ml), and an isotype-matched MAb of irrelevant specificity. In the legend at the top of the figure, for each column "+" indicates use of this treatment, and "-" indicates that the treatment was not used. After five hours incubation of cells, RNA was purified and subsequently probed with bovine tissue factor cDNA. Experiments were repeated twice to confirm results.
PART 3
ANALYSIS OF THE CD14 RECEPTORS ASSOCIATED WITH BOVINE ALVEOLAR MACROPHAGES AND PRESENT IN BOVINE SERUM

Abstract

Introduction

Materials and Methods

Bovine alveolar macrophages
Immunoprecipitation of $^{125}$I-labeled macrophage surface protein
Preparation of the CD14 probe and macrophage RNA
Hybrid-selected translation
Western blot analysis

Results and Discussion

Acknowledgements

References

Figures 3-1 to 3-3
Abstract

Previous studies have suggested the existence of a bovine homolog of the membrane-associated CD14 receptor (mCD14) on macrophages, and functional similarity of bovine mCD14 to receptor activity reported in other species. Bovine alveolar macrophages (bAM) reportedly possess two mRNA transcripts of 1.5 and 3.1 kb for CD14, rather than a single 1.5 kb transcript as reported for other species. The purpose of this study was to determine the molecular mass of the bovine CD14 receptor, and to determine if the two mRNA transcripts for bovine CD14 yield either a single or two different gene products. Culture supernatant from ^{125}I-surface-labeled bAM was examined for the existence of bovine CD14 using SDS-PAGE and autoradiography. A single protein band of 49 kD was immunoprecipitated from the supernatant using anti-CD14 monoclonal antibodies (MAb). Macrophage-derived mRNA was subjected to hybrid-selection using a human CD14 cDNA probe immobilized on a nitrocellulose filter. The resultant, selected bovine mRNA was then utilized for in vitro translation, and protein of 38-40 kD was synthesized. This size is consistent with an unglycosylated CD14 receptor protein. Protein was also synthesized from total RNA by in vitro translation, and was immunoprecipitated with anti-CD14 monoclonal antibodies. A doublet-band of protein was seen at 38 kD using SDS-PAGE and autoradiography. These results demonstrate that a receptor consistent with mCD14 is present on bovine macrophages, and the form of the receptor released into supernatants is 49 kD. Western blot analysis
performed on fractionated bovine serum samples with anti-CD14 MAb revealed immunoreactivity with a 50-55 kDa protein; a size consistent with sCD14.

Introduction

In most gram-negative bacterial diseases, the acute immunoinflammatory response is initiated by bacterial lipopolysaccharide (endotoxin; LPS). LPS-related inflammatory disease results from excessive synthesis of a complex combination of cytokines, eicosanoids, and other mediators that are released from LPS-stimulated cells such as macrophages (1-3). Stimulatory pathways and intracellular mediators activated by LPS have been demonstrated and appear to include components such as tyrosine kinases (4-5), MAP kinase P38 (6), and NFκB (7). Stimulation is also highly dependent upon the involvement of several important membrane-associated and extracellular LPS-binding proteins. These include LPS-binding protein (LBP), soluble CD14 (sCD14), and membrane CD14 (mCD14) (8-13). Current understanding indicates that bacterial LPS binds LBP with high affinity, and the LPS-LBP complex interacts with either sCD14 or the mCD14 receptor. The glycosylphosphatidylinositol-linked (GPI) mCD14 receptor transfers LPS to another receptor/signal transducer located on the membrane, with resultant intracellular signalling and cellular activation. A recent study demonstrated that LPS forms a ternary complex with LBP and mCD14, initiating both signal transduction and internalization/clearance (14).
In order to better characterize the pathways utilized by LPS in other domestic species such as cattle, monoclonal antibodies and other probes have previously been employed to describe the existence of a functional mCD14-like homologue on bovine alveolar macrophages (15-16). Two bovine mRNA transcripts of 1.5 and 3.1 kb were demonstrated which hybridized with a human CD14 cDNA probe (15), notwithstanding the fact that human CD14 has a single mRNA transcripts of 1.5 kb. In our present study, we demonstrate that the native bovine CD14 receptor molecule released from macrophages into culture supernatants is a 49 kD protein. We also show that an in vitro-translated product derived from bovine CD14 mRNA appears as 38-40 kD protein, which is consistent in size with an unglycosylated CD14 receptor. Western blot analysis detected a 50-55 kDa protein in bovine serum fractions. These data suggest that although it appears that bAM make two mRNA transcripts of CD14, present evidence does not support the concept that two distinct proteins are synthesized.

**Materials and Methods**

**Bovine Alveolar Macrophages**

Bovine alveolar macrophages (bAM) were collected and isolated from lungs obtained at a local abattoir using similar methodology as has been previously described (16). Lavage fluid with cells was filtered through sterile gauze and the cells were pelleted by centrifugation. Cells were then
resuspended and washed twice in sterile, pyrogen-free saline before final resuspension in Dulbecco’s Modified Eagles’ Media (DMEM). The percentage of macrophages determined by Wright-Giemsa stained cytocentrifuge preparation was greater than 90%, and viability determined by trypan blue dye exclusion was also greater than 90%.

**Immunoprecipitation of 125I-Labeled Macrophage Surface Proteins**

Surface proteins on bAM (5 X 10⁶ cells) were radiolabeled with Na¹²⁵I (sp. activ. ≥ 400 mCi/ml; ICN, Costa Mesa, CA) using IODO-BEADS™ iodination Reagent (PIERCE, Rockford, IL), then washed three times with physiologic buffer to remove unbound radioisotope. The radiolabeled cells were subsequently treated with 100 ng/ml of phorbol 12-myristate 13-acetate (PMA; Sigma Chemical Co., St. Louis, MO) in serum-free DMEM at 37°C for 2 hours to enhance shedding of mCD14 into the medium. The supernatant was collected and then concentrated with a Centricon-10 spin column (10 kD cutoff; Amicon, Danvers, MA). The sample containing ¹²⁵I-labeled membrane proteins was used for immunoprecipitation with immobilized protein G-coupled anti-CD14 MAb My4 (10 µg/0.2 ml; Coulter Immunology, Hialeah, FL) and MAb UCHM-1 (10 µg/0.2 ml; Sigma). A protein G-coupled murine myeloma IgG MAb (Organon Teknika, Malvern, PA) was included as a control. Eluate was separated with 10% SDS-PAGE gel for autoradiography.
Preparation of the CD14 Probe and Macrophage RNA

Plasmids encoding cDNA for human CD14 and *E. coli* MC1061/p3 bacteria were generously provided by Dr. Brian Seed, Department of Molecular Biology, Massachusetts General Hospital, Boston. The purified human CD14 cDNA probe had previously been shown to hybridize with bovine CD14 mRNA in Northern blots (15). Transformation of the CD14 cDNA-containing plasmids into bacteria was carried out using the CaCl$_2$ heat-pulsed method, and double selection was performed with tetracycline and ampicillin LB-plates. Total cellular RNA was purified from bAM using phenol-guanidine isothiocyanate (TRIZOL Reagent, Life Technologies, Gaithersburg, MD) according to the manufacturer's instructions. The RNA concentration was determined by measurement of $A_{260}$, and the $A_{260/280}$ ratio of macrophage RNA used in these experiments was greater than 1.8.

Hybrid-Selected Translation

cDNA for CD14 was denatured by heating prior to use for hybrid-selected translation (17,18), then immobilized on a nitrocellulose membrane using a slot-blot apparatus (Life Technologies, Gaithersburg, MD) following by UV cross-linking. Portions of the membrane with immobilized CD14 cDNA were cut out, then prehybridized in a 1.5 ml Eppendorf tube at 37°C for 3 hours with a prehybridization buffer containing 50% formamide, 0.01% Pipes (pH 6.4), 0.4 M NaCl, and 300 $\mu$g/ml tRNA. After the reaction mixture was
aspirated, the sections of membrane were subsequently hybridized at 37°C for 16 hours with hybridization buffer (prehybridization buffer without tRNA) containing 300-600 μg bovine macrophage total RNA, which had been previously denatured by heating at 67°C for 10 minutes followed by rapid cooling on ice. The total volume for this reaction was 100-150 μl. After hybridization, the membranes were washed 10 times by vortexing with a buffer containing 150 mM NaCl, 15 mM sodium acetate, and 0.5% sodium dodecyl sulfate (SDS) with incubation for one minute at 60°C during wash steps. Three additional washes were then performed using 2 mM EDTA at 25°C. Hybrid-selected mRNA was then eluted from the membrane blots with 0.3 ml of boiling DEPC-treated water for 1 min. The mRNA-containing solution was quick-frozen in a dry ice bath for storage prior to the following steps.

Selected mRNA was co-precipitated with 10 μg tRNA using sodium acetate and ethanol. Prior to translation, the RNA mixture in 70% ethanol was pelleted at 12,000 rpm for 10 min at 4°C, dried, then dissolved in 10 μl of water. The translation reaction was carried out in reticulocyte lysate using a nuclease-treated rabbit reticulocyte lysate system (Pharmacia, Piscataway, NJ), and the positive control consisted of chloramphenicol acetyltransferase (CAT) mRNA (Life Technologies, Gaithersburg, MD). Twenty μCi of 35S-L-methionine (specific activity 1,174 Ci/mmmole; ICN, Irvine, CA) was added into the 25 μl translation reaction. Following a two hour incubation at 30°C, pancreatic RNase A (0.1 mg/ml, Sigma) was added for 15 min at 30°C. Five μl of each
resulting sample was diluted with 15 μl loading buffer, heated to 70°C, then loaded on 10% SDS-PAGE for separation followed by autoradiography. The translated samples were also subjected to immunoprecipitation using protein G-coupled anti-CD14 MAb My4 (20 μg/0.2 ml) or control antibodies of irrelevant specificity and of the same isotype and concentration, and the eluates were subjected to SDS-PAGE. The resulting gels were dried, then autoradiography was performed using preflashed Kodak X-Omat XAR-5 film at room temperature for an appropriate period.

**Western Blot Analysis.**

Blots performed on samples of whole serum do not allow visualization of immunoreactivity, probably due to the relatively low concentration of sCD14 in serum compared to other components, such as bovine serum albumin. We used samples of fractionated bovine serum prepared using ion-exchange chromatography (16) and then concentrated the samples approximately ten-fold before subjecting 30 μg of the serum proteins to SDS-PAGE (10%, reducing conditions). Serum proteins from the 0.2, 0.3-0.5, and 1.0 M NaCl ion-exchange-eluted protein pools were heated at 70°C for two min prior to electrophoresis, and then transferred to a 0.45 μM nitrocellulose membrane with a semidry blotting apparatus. The preparation was blocked by incubation with 2% bovine serum albumin at 22°C for 2 hour, then incubated with anti-CD14 MAb 60bca (2 μg/ml) in Tris-buffered solution (TBS) for 1 hours at
22°C, followed by three washes with TBS. Blots were then incubated with the second antibody (goat anti-mouse IgG Fc) conjugated with alkaline phosphatase in TBS with 0.02% tween-20 for 1 hours at 22°C. The second antibody had been absorbed with bovine, equine, and human serum proteins by the manufacturer. Following three washes with TBS, immunoreactivity was visualized with alkaline phosphatase substrates.

Results and Discussion

It is generally recognized that the CD14 receptor plays a significant role in presentation of bacterial LPS to cells such as macrophages, neutrophils, and endothelial cells (8). The soluble form (sCD14) of this receptor in plasma and the membrane-bound form (mCD14) on the surface of macrophages and neutrophils appear to function in a somewhat different manner (10,13), but both serve in acquisition of LPS by receptive cells. The human mCD14 receptor has been described as a GPI-linked protein of 53 kD in SDS-PAGE (20). It has been demonstrated that either one or two protein bands ranging from 48-55 kD and corresponding to CD14 appear in supernatants prepared from human monocytes and neutrophils, depending upon culture conditions (21,22). Two forms of sCD14 are found in human plasma and may be released by human monocytes, and have been designated sCD14α (low M,) and sCD14β (high M,) (23). Whether or not most or all of the sCD14 in plasma results from cellular shedding of monocyte/macrophage mCD14 is unresolved.
We employed anti-human CD14 monoclonal antibodies (My4, UCHM1) for immunoprecipitation of radiolabeled proteins from bovine macrophage supernatants, followed by SDS-PAGE and autoradiography (Figure 3-1). Our result reveals the presence of a signal associated with a 49 kD protein in the two lanes associated with the anti-CD14 MAbs ("My4", "UC"), but no protein of this size in the lane with the control antibody (right, "IgG_2"). Several weaker signals of probable nonspecific nature appear in all three lanes. This data suggests that the anti-CD14 MAbs immunoprecipitate a bovine macrophage surface protein with a mass of 49 kD, which is compatible with the reported size range of human and murine mCD14.

Although several molecular sizes of human CD14 have been reported, all forms may come from a common 50 kDa precursor (23), which is supported by the presence of a single transcript of CD14 mRNA (1.5 kb) identified in human cells. Curiously, bovine alveolar macrophage CD14 mRNA appeared as two transcripts of 1.5 kb and 3.1 kb (15). This observation prompted us to investigate whether bovine macrophages synthesize two distinct CD14 molecules from the two mRNA transcripts. Hybrid-selection of bovine mRNA followed by in vitro translation was carried out, and is shown in Figure 3-2A. It appears that bovine mRNA selected for its complementarity to human CD14 cDNA (lane "Sel", right-center) primarily results in synthesis of protein with a molecular mass of 38-40 kD. This size is similar to the reported mass for deglycosylated, recombinant human CD14 (24).
(negative control, left) did not result in meaningful protein synthesis, CAT (positive control, center-left) yielded the expected band of protein, and total unselected macrophage RNA ("Total", right) resulted in the expected smear of numerous proteins.

The anti-CD14 monoclonal antibody My4 was then used to precipitate immunoreactive, translated protein from the mixture of translated proteins resulting from use of total RNA. The immunoprecipitated protein was subjected to SDS-PAGE and autoradiography, and is seen in Figure 3-2B. A doublet-band of approximately 38 kD is seen as a result of anti-CD14 antibody precipitation (left, "My4"), but not with use of control antibody (right, "IgG2a"). This size (38-40 kD) is compatible with the hybrid-selected protein seen in Figure 3-2A ("Sel"). The other weaker protein band in Figure 3-2B of mass greater than 50 kD is seemingly nonspecific and also variably appears in other lanes of Figures 3-2A and 3-2B. These data indicate that although bovine CD14 mRNA appears as two forms (1.5 and 3.1 kB) upon Northern-blot analysis, protein of 38-40 kD is translated. The significance of the two forms of mRNA remains unresolved.

In summary, a protein consistent with bovine mCD14 appears to be released from the surface of alveolar macrophages, and has an apparent molecular mass consistent with that reported for human mCD14. The existence of two mRNA transcripts for bovine CD14 has been previously reported (15), but our data shows an in vitro-translated protein of 38 to 40 kD.
which resulted from use of the two hybrid-selected mRNA transcripts of bovine CD14 mRNA. Two distinct bovine CD14 proteins are apparently not synthesized from the two mRNA transcripts.

In addition, we demonstrated the existence of soluble CD14 in bovine serum by western blot analysis. The blots were performed on samples of fractionated bovine serum using anti-CD14 MAb 60bca (2 μg/ml) (Figure 3-3). Serum proteins from the 1.0 M NaCl ion-exchange chromatography-eluted proteins failed to react with the antibody (left), but bovine serum proteins of 50-55 kDa showed strong immunoreactivity in the 0.3-0.5 M NaCl-eluted fraction (right). This immunoreactivity indicated that the presence of sCD14 in the 0.3-0.5 M bovine serum fraction. In addition, endothelial cell cytotoxicity assays were conducted using the serum chromatography fractions, and the 0.3-0.5 M NaCl-eluted fraction (Figure 3-3, center lane, which had the greatest reactivity of a CD14-like protein) also elicited the greatest quantity of LPS-mediated cell death (data not shown).

Collectively these results support the concept that the bovine mCD14 and sCD14 receptors have a similar molecular weight to those CD14 receptors of humans, rabbits, and mice.

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References


1990. CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein. Science 249:1431-1433.


Figure 3-1. Immunoprecipitation of $^{125}$I-labeled surface proteins from bovine alveolar macrophages with anti-CD14 monoclonal antibodies. Anti-human CD14 monoclonal antibodies My4 (left) and UCHM1 (center, "UC"), and a control monoclonal of irrelevant specificity (right, "IgG$_2$") were used to immunoprecipitate macrophage-derived, radiolabeled surface proteins. Precipitates were separated with SDS-PAGE and visualized with autoradiography. The arrow indicates a signal associated with a 49 kD protein in the two lanes associated with the anti-CD14 MAbs ("My4", "UC").
Figure 3-2. Hybrid-selection of bovine mRNA using CD14 cDNA followed by in vitro translation. (A). Synthesized proteins resulting from in vitro translation were separated with SDS-PAGE and visualized with autoradiography, employing \(^{35}\)S-methionine as a label incorporated into translated protein. "tRNA" (left, negative control) did not result in substantial protein synthesis; "CAT" (left-center, positive control) produced the expected band of protein; "Sel" (right-center, bovine mRNA hybrid-selected for its complementarity to human CD14 cDNA) results primarily in synthesis of protein with a molecular mass of 38-40 kD; "Total" (right, total unselected macrophage RNA) resulted in a smear of numerous translated proteins. (B) Anti-CD14 monoclonal antibody My4 was used to precipitate immunoreactive, translated protein from the mixture of translated proteins resulting from use of total RNA (refer to lane "Total", right, Figure 2A, above). Immunoprecipitated protein was subjected to SDS-PAGE and autoradiography. The arrow indicates a doublet-band of approximately 38 kD as a result of anti-CD14 antibody precipitation (left, "My4"), which does not appear with use of control antibody (right, "IgG\(_{2b}\)").
Figure 3-3. Western blot analysis of bovine serum fractions. Blots were performed on samples of fractionated bovine serum using anti-CD14 MAb 60bca (2 μg/ml). Serum proteins from the 1.0 M NaCl ion-exchange chromatography-eluted proteins appears in the left lane, bovine serum proteins from the 0.3-0.5 M NaCl-eluted fraction are in the center lane, and proteins from the 0.2 M NaCl-eluted fraction appear in the right lane.
PART 4

CD14 AND BOVINE LIPOPOLYSACCHARIDE-BINDING PROTEIN PARTICIPATE IN LPS-MEDIATED ACTIVATION AND CYTOTOXICITY OF BOVINE ENDOTHELIAL CELLS IN VITRO

Abstract

Introduction

Materials and Methods

Endothelial cells
Tissue factor assay
Cytotoxicity assay
Preparation of bovine LBP
Anti-CD14 immunofluorescence analysis of endothelial cells
Northern blot analysis
Media and reagents

Results

Tissue factor expression by LPS-stimulated bovine endothelial cells is dependent upon bovine serum
Anti-CD14 antibodies decreases tissue factor expression by bEC
Anti-CD14 antibodies reduce LPS-mediated cytotoxicity to bEC
rsCD14 supplants serum in LPS-mediated cytotoxicity to bEC
bLBP synergizes with rsCD14 in LPS-mediated cytotoxicity to bEC
Absence of membrane-associated CD14 on bEC

Discussion

Acknowledgements

References

Figures 4-1 to 4-6
Abstract

Bacterial endotoxin is a potent proinflammatory agent which has stimulatory effects on many cell types, including vascular endothelial cells. Bovine endothelial cells and fetal bovine serum are widely-used for investigations involving vascular endothelial cells, and we now demonstrate that endotoxin-mediated activation and cytotoxicity to bovine endothelial cells in vitro is dependent on CD14. Endotoxin-mediated activation of endothelial cells was quantitated by tissue factor expression using S-2222 and an activated factor X-related chromogenic assay. Concentrations of 0.4-2.0% fetal bovine serum in the culture medium dramatically promoted LPS-induced tissue factor expression on bovine endothelial cells, implying that serum factors participate in the effect on endothelial cells. When added to the incubation medium, anti-CD14 MAb (60 bd/bca, 20 μg/ml) inhibited tissue factor expression, but control antibodies did not. The MTT colorimetric assay was used to assess LPS-mediated cytotoxicity to the endothelial cells. We found that either serum or recombinant soluble CD14 (rsCD14) was required for LPS-mediated cytotoxicity to endothelial cells, and that anti-CD14 MAb inhibited cytotoxicity. In addition, purified bovine LPS-binding protein (20 ng/ml) greatly enhanced the cytotoxic effect of LPS plus rsCD14. Membrane-associated CD14 could not be detected on the surface of endothelial cells using immunofluorescence technique, and northern blot analysis failed to detect any bovine endothelial cell-associated mRNA for CD14. These results collectively
suggest that soluble bovine serum factors including sCD14 and LBP participate in endotoxin-mediated activation and cytotoxicity of bovine endothelial cells, and that the serum factors may facilitate presentation of LPS to endothelial cells by a similar pathway to that which has been documented for human cells.

Introduction

Bacterial lipopolysaccharide (LPS, endotoxin) is well-known for its formidable proinflammatory effects in mammals (24, 25, 32). When liberated in sufficient amounts from gram-negative bacteria, LPS causes symptoms and adverse sequela in man and animals that include fever, coagulopathies, shock, and in some cases multiple organ failure and death (23, 25, 28, 34). The suffering and economic loss associated with endotoxemia have generated interest and prompted noteworthy research during the past two decades into the mechanisms of action of LPS.

Endotoxin is unlike many other bacterial toxins in that it is nearly ubiquitous in the environment (34). Considering the high risk for exposure, it is therefore not surprising that intrinsic biologic mechanisms have evolved in mammals to deal with this toxin. A number of plasma proteins including lipoproteins, serum albumin, anti-LPS antibodies, soluble CD14 receptor (sCD14), and lipopolysaccharide-binding protein (LBP) are now known to interact with LPS and may modify its biologic activity (2, 14, 36, 37, 48). The best-understood interaction of this type is the recently-elucidated mechanism
of binding of LPS to cells of myelomonocytic origin via interaction with serum LBP and membrane-associated CD14.

LBP has been identified in the normal sera of humans (37), rabbits (45), and mice (10), and is an acute-phase protein which increases in concentration in response to acute inflammatory stimuli. LBP in serum binds LPS with high affinity (37,46), and the resultant LBP-LPS complex binds to membrane-associated CD14 receptors (36,50) on the surface of monocytes, macrophages, and neutrophils. This interaction enables these leukocytes to respond to much lower concentrations of LBP-LPS complexes compared to LPS alone, and lowers the LPS threshold for generation of proinflammatory cytokines such as TNFα and IL-6 (6,19,37). Resultant synthesis of proinflammatory and immunomodulatory cytokines at low concentrations of LPS may be beneficial to the host, but overproduction of these cytokines can be detrimental or fatal. Although a role for LBP and membrane-associated CD14 in LPS-mediated activation of myelomonocytic cells is now well-established, a role for sCD14 and LBP in activation of endothelial cells has only recently been proposed (14,31).

In addition to the 53-55kD, membrane-associated CD14 molecule with its glycerophosphatidylinositol anchor, soluble forms of CD14 (sCD14) are also normally present in human plasma (7,13). sCD14 interacts with LPS, and it has recently been proposed that LPS-LBP complexes shuttle LPS to sCD14, and the LPS-sCD14 complexes then bind to specific receptors on endothelial and
epithelial cells (31). In addition to the activation-associated role of sCD14 with endothelial cells, it is also conceivable that sCD14 may compete for LPS and render LPS unavailable for monocyte activation, and therefore play contrary roles depending on the cell type.

When vascular endothelial cells are stimulated or damaged by LPS, endothelial cells may initiate profound proinflammatory or deleterious effects on the host. It has long been known that LPS will damage or activate endothelial cells derived from humans or other species, that LPS may induce cytotoxic effects on bovine endothelial cells, and that the presence of serum potentiates the effects of LPS on endothelial cells (12,20,21,41). Researchers have recently demonstrated that human recombinant sCD14 can supplant the requirement for serum in LPS-cytotoxicity experiments utilizing cultured human and bovine endothelial cells (1,8,14,31). Although sCD14 in combination with LPS was sufficient to induce effects on EC, the presence of LBP was also needed for the endothelial cell-associated effects to be seen at low, physiologically-relevant concentrations of LPS (1-10 ng/ml) (14,31). A specific receptor on endothelial cell-surfaces which binds the LPS-sCD14 or LPS-LBP-sCD14 complexes has not yet been identified. These findings have elucidated an important pathway in LPS-initiated human endothelial cell activation, and assist scientists and clinicians in better understanding mechanisms of LPS-endothelial cell interactions in septic patients.

Bovine endothelial cells (bEC) cultured in vitro are widely-utilized as a
model system by many investigators to examine various biomedical questions regarding the vascular barrier. bEC are similar to human EC in that both types of cells may be activated by LPS in combination with serum factors, but bEC also suffer cytotoxic effects from LPS that human EC do not exhibit. Despite the widespread use of bEC and fetal bovine serum (FBS) in scientific research, investigators have not established evidence for the existence of a bovine homolog of sCD14 in FBS, nor has the presence/absence of membrane-associated CD14 on bovine vascular endothelial cells been examined. In this study we have confirmed the effect of LPS and bovine serum factors on activation and cytotoxicity to bEC, and have found that anti-CD14 monoclonal antibodies can reduce or abolish activation of bEC and the LPS-associated cytotoxic effects in the presence of either FBS or rsCD14. In addition, we have found that LBP purified from bovine serum enhanced the cytotoxic effect of rsCD14 and LPS. We have found no evidence for membrane-associated CD14 on the surface of bEC, suggesting that the role of CD14 in LPS-endothelial cell interactions may be limited to that of a soluble receptor.

**Materials and Methods**

**Endothelial Cells**

Primary bovine endothelial cell (bEC) cultures were established from adult bovine pulmonary arteries using standard collagenase digest methodology combined with subcloning of endothelial cell colonies (38). The cells were
identified as bEC by typical cobblestone morphology using phase-contrast microscopy, the presence of factor VIII-related antigen (35), and positive staining (uptake) with acetylated low-density lipoprotein (Dil-Ac-LDL; 1,1'–dioctadecyl-3,3',3'-tetramethyl-indocarbocyanine perchlorate). Acetylated low-density lipoprotein is known to be selectively internalized by mononuclear phagocytes and endothelial cells without affecting cell viability, and emits red fluorescence (30,40). The bECs used in all experiments were less than 20 passages and maintained in DMEM plus 10% fetal bovine serum (FBS) and 2 mM L-glutamine. Culture media was removed and cells were washed three times before use in subsequent experiments.

**Tissue Factor Assay**

Tissue factor (TF) expression was used to assess endothelial cell activation, and was quantitated with a colorimetric assay based on digestion of substrate S-2222 by activated factor X (42). BECs were plated at 30,000 cells/well in 96-well flat-bottom tissue culture plates with DMEM plus 2 mM L-glutamine and allowed to adhere overnight. Endotoxin (LPS) from *E. coli* (serotype O55:B5; 5 or 50 ng/ml) was added in the presence of various concentrations of FBS for a period of 6 hours. When using antibodies, cells in the plate were preincubated with the antibody for 30 min before addition of LPS. At the end of the incubation period with LPS, the plates were washed with Hank’s balanced salt solution (HBSS), followed by addition of 100 µl/well
of HBSS (without phenol red) containing 200 μg/ml of substrate S-2222, and 1 unit factor VII/ml of Proplex T (consisting of factor II, VII, IX, and X). After incubation at 37°C for 120 min, the optical density (405 nm) was quantitated using an automated Bio-Tek microplate reader. Blank controls consisted of wells without LPS, and all background values were subtracted before data analysis. Each treatment variable was performed in quadruplicate, and experiments were repeated at least twice to confirm results.

**Cytotoxicity Assay**

Bovine ECs in DMEM medium were seeded into 96-well plates at 20,000 cells/well and allowed to adhere overnight. FBS, rsCD14, or/and bLBP were added to different wells, murine anti-human CD14 MAb (60bd, 60bca, or My4) or an irrelevant isotype-specific control monoclonal antibody (murine IgG1 or IgG2b) was added, and 30 min later LPS from *E. coli* was added. After 24 hour incubation at 37°C in 5% CO₂, the MTT tetrazolium cytotoxicity assay (26) was used to quantitate cell death due to LPS. 10 μl of MTT at a concentration of 5 mg/ml in phosphate-buffered saline (PBS) was added to each well, and further incubated for 4 hours at 37°C. After aspirating the supernatant from each well, 100 μl of isopropanol with 0.04 N HCl and 25 μl of 3% sodium dodecyl sulfate (SDS) were added to all wells. When the dark blue formazan had dissolved, plates were read on a Bio-Tek microplate reader at a wavelength of 570 nm. Control wells consisted of untreated BEC. Cytotoxicity was
determined as:  % dead cells = (1-(O.D.\textsubscript{test}/O.D.\textsubscript{control media})) \times 100.

**Preparation of Bovine LBP**

Pooled bovine serum collected from normal, healthy cows was fractionated essentially as described for rabbit serum (45) and bovine serum (17). The serum fractions were screened for purity and presence of a 60-65 kD protein using 10% SDS-PAGE with reducing conditions, followed by silver staining. Qualitatively similar fractions were pooled, and the identity of the isolated protein (bLBP) confirmed by N-terminus sequencing (17) directly from the unblotted chromatography fractions. Samples were lyophilized and stored at -70°C until use.

**Anti-CD14 Immunofluorescence Analysis of Endothelial Cells**

Possible surface expression of CD14 on bEC was assessed using monoclonal antibody 60bd and indirect immunofluorescence technique. Briefly, bEC were cultured on 8-chamber plastic slides, washed twice with isotonic buffer, then fixed for 10 minutes using a 1:1 mixture of absolute ethanol:acetone. After washing, cells were blocked for 20 min. with a mixture of 1% heat-inactivated bovine serum albumin and 100 μg/ml of irrelevant murine IgG. The solution was then tapped off without washing, and the anti-CD14 primary antibody (60bd, 20 μg/ml) was applied to some treatment groups and incubated with cells for 30 min at 37°C. After washing twice with PBS,
cells were then blocked for 10 min. with 1% heat-inactivated bovine serum albumin plus 1% heat-inactivated goat serum. Cells were finally incubated with FITC-labeled goat anti-mouse IgG for 30 min. at 37°C. The cell preparation was then washed three times with PBS, cover slipped, and immediately viewed on a fluorescence microscope.

**Northern Blot Analysis**

Total cellular RNA was extracted using 4 M guanidine thiocyanate/0.1 M Tris-HCl (pH 7.5)/1% 2-mercaptoethanol solution, followed by centrifugation in 6.1 M CsCl/25 mM NaAc (pH 5.2)/10 mM EDTA solution (18). Resulting RNA pellets were redissolved and precipitated by centrifugation from a 70% ethanol/0.3 M NaAc (pH 5.2) solution. Pellets were rinsed with 70% ethanol, dried in a vacuum desiccator, and dissolved in 100 mM sodium phosphate buffer (pH 7.0). RNA concentration was determined by measurement of UV absorbance at 260 nm. RNA was fractionated by electrophoresis in a 1% agarose gel, transferred by capillary action to nylon membranes (43), and immobilized by UV irradiation with a UV cross linker (Hoefer, San Francisco). Blots were pre-hybridized for 3 hours at 42°C in 50% formamide, and hybridized overnight at 42°C with the 32P-labeled cDNA probe for human CD14. The hybridized blot was washed twice in 2 X SSC at room temperature, and then three times in 0.1 X SSC/0.1% SDS at 55°C. Blots were then incubated with preflashed Kodak X-Omat XAR-5 film and an intensifying screen at -70°C
for an appropriate period (1-3 days). Uniformity of RNA loading on the gels was verified by visual assessment (photography) of total RNA loaded on agarose gels. Blots were repeated to confirm results.

**Media and Reagents**

The recombinant human sCD14 was generously provided by AMGEN, Inc., Thousand Oaks, CA. Dulbecco’s Modified Eagle’s Medium (DMEM) was purchased from Whittaker MA Bioproducts, Walkersville, MD. Hank’s Balanced Salt Solution (HBSS) without phenol red from GIBCO Laboratories, Grand Island, NY, and fetal bovine serum (FBS) was obtained from HyClone Laboratories, Inc., Logan, UT. FBS contained 0.6 unit endotoxin/ml and 32 mg protein/ml, according to the manufacturer. Pooled normal bovine serum contained 79 mg protein/ml. LPS used in this study was from *Escherichia coli* serotype O55:B5, Sigma Chemical Co., St. Louis, MO. MTT tetrazolium was obtained from Sigma Chem. Co., St. Louis, MO. Chromogenic substrate B2-Ile-Glu-Arg-P-nitroanilide (S-2222) was from Kabi Pharmaceuticals, Franklin, OH; a coagulation factor concentrate (Proplex T) was obtained from Travenol Labs, Inc., Glendale, CA. Murine anti-CD14 monoclonal antibody (MAb) 60bd (IgG1, provided as ascites) was a generous gift from Dr. Robert F. Todd III, Division of Hematology and Oncology, University of Michigan Medical School, Ann Arbor, Michigan. Anti-human CD14 MAb 60bca (IgG1) is from a line established from the 60bd clone, is similar in function to 60bd, and was
purchased from ATCC, Rockville, MD. Both antibodies were purified from ascites using an ImmunoPure (G) IgG Purification Kit (Pierce, Rockford, IL) and stored without preservative at -70°C until use. My4, another anti-human CD14 murine monoclonal antibody (IgG2b) that was used in some assays, was purchased from Coulter Immunology (Hialeah, FL). The two control isotype-matched MAb used in these experiments were mouse IgG1 (Kappa), derived from MOPC 21, a mineral oil-induced plasmacytoma, and mouse IgG2b (Kappa), derived from MOPC 195, also a mineral oil-induced plasmacytoma. Neither of these have any known hapten or antigen binding activity; they were from Organon Teknika Corp., Malvern, PA. The cDNA probe for human CD14 was a kind gift of Dr. Brian Seed, Department of Molecular Biology, Massachusetts General Hospital, Boston, MA. Other reagents for the Northern blots were purchased from GIBCO/BRL, Grand Island, NY.

Results

Tissue Factor Expression by LPS-Stimulated Bovine Endothelial Cells is Dependent Upon Bovine Serum

The stimulatory activity of LPS on bEC was dependent on the presence and concentration of bovine serum (baseline on graph; Figure 4-1). At the concentration tested, LPS alone had no measurable effect on tissue factor expression by the bEC. FBS or NBS at the concentration of less than 10% did not stimulate obvious tissue factor expression in the absence of addition of
LPS. Increasing concentrations of either FBS or normal adult bovine serum (NBS) in combination with LPS had a potent stimulatory effect, with 0.4 - 2.0% serum causing maximal potentiation. At the highest concentration tested (10%), both FBS and NBS appeared to lose some of the LPS-potentiating effect on tissue factor expression by bEC.

**Anti-CD14 Antibodies Decrease Tissue Factor Expression by bEC**

Inhibition of tissue factor expression occurred in the presence of the anti-CD14 MAb 60bd (Figure 4-2). The amount of inhibition was dependent on the concentration of FBS present, however, and the effect was absent at concentrations of FBS ≥ 5%. We also tested another commercially available anti-CD14 MAb (My4) in this tissue factor expression assay, and found that My4 also inhibited LPS-stimulated, FBS-dependent tissue factor expression of bEC by 65% in the presence of 1% FBS (data not shown).

**Anti-CD14 Antibodies Reduce LPS-Mediated Cytotoxicity to bEC**

The MTT cytotoxicity assay was used to assess potential effects of anti-CD14 MAb on LPS-mediated toxicity to bEC *in vitro*. After 24 hours of incubation with LPS ± FBS, we found that increasing concentrations of FBS augmented the cytotoxic potential of LPS (Figure 4-3). Anti-CD14 MAb 60bca at 20 μg/ml abolished the cytotoxic effect of LPS at FBS concentrations of 0.5%, and reduced the cytotoxicity from 73.0 ± 7.9% (mean ± SD; n=4),
to 24 ± 1.0% (n = 4) at 1.0% FBS, but the protective effect of the MAb was lost at higher concentrations of FBS. The control MAb IgG1 did not produce inhibition of LPS-mediated, bovine serum dependent cytotoxicity of bEC. A dose-dependent assay for anti-CD14 MAb 60bca was carried out (Figure 4-3B), and demonstrated an antibody-dependent effect.

**rsCD14 Supplants Serum in LPS-Mediated Cytotoxicity to bEC**

We used rsCD14 in place of FBS for LPS-mediated bEC cytotoxicity assays (Figure 4-4), and rsCD14 at concentrations ≥ 1 μg/ml was able to cause LPS-mediated bEC cytotoxicity. This effect was inhibitable by anti-CD14 antibody. We also tested another anti-CD14 MAb, My4, and obtained similar data (not shown). With a concentration of 1 μg/ml of rsCD14, the induced cytotoxicity to bEC was dependent on the concentration of LPS (Figure 4-4B). 10 ng/ml LPS plus rsCD14 did not cause notable cytotoxicity of bEC, but 50 ng/ml LPS was injurious.

**bLBP Synergizes with rsCD14 in LPS-Mediated Cytotoxicity to bEC**

We used bovine LBP in combination with rsCD14 and LPS to find out whether LBP contributes to this process. 10 ng/ml LPS was added to a variety of treatments, shown in Figure 4-5A. Note that 10 ng/ml LPS plus rsCD14 (1 μg/ml) did not produce notable cytotoxicity (see also Figure 4-4B). Bovine LBP (20 ng/ml) alone or with LPS (10 ng/ml) had no measurable effect on the cells.
However, bovine LBP (20 ng/ml) was required for a significant cytotoxic response to LPS (10 ng/ml) plus rsCD14. Even relatively high concentrations of rsCD14 (2.5 μg/ml) plus LPS (10 ng/ml) required the presence of bovine LBP. In contrast, 35 ± 1.7% (n=4) cytotoxicity was observed with a combination of 10 ng/ml LPS, bLBP, and 100 ng/ml rsCD14. This result, associated with the result in Figure 4-4B in which 50 ng/ml LPS and 1 μg/ml rsCD14 were required for measurable cytotoxicity, indicates that bLBP enhanced the effect of LPS-sCD14 on endothelial cells. Furthermore, this effect was completely blocked by anti-CD14 antibody.

Absence of Membrane-Associated CD14 on bEC

We used indirect immunofluorescence technique and fluorescence microscopy to investigate the presence of membrane-associated CD14 on the surface of bEC, and failed to detect CD14-associated fluorescence on the cells (data not shown). Although this technique should detect highly-expressed receptors on cell surfaces, it may lack the sensitivity to detect low copy number receptors. We also performed Northern blot analysis using an anti-human cDNA probe on total cellular RNA from bEC, and human and bovine monocytes/macrophages as controls (Figure 4-6). The human monocyte control exhibited the expected band at 1.5 kb, and the bovine alveolar macrophages also exhibited a weaker band of this size. The bovine macrophages also show an unexpected band at 3.1 kb. However, mRNA from
bEC did not hybridize with the probe, indicating that mRNA for CD14 is undetectable in bovine endothelial cells when employing this technique.

**Discussion**

Vascular endothelial cells play an important role in regulation of vascular homeostasis, and damage to or activation of these cells can result in detrimental changes in humans and animals. Endothelial cells may play specific roles in the pathophysiology of Gram-negative bacterial sepsis, particularly in the manifestation of coagulopathies, shock, and organ failure (5,20,22,27,49). Because bEC are used in vitro to study endothelial-cell related phenomena, it is important to better define the roles of soluble serum factors and LPS on these cells. In addition, widespread use of FBS in laboratory tissue culture makes it desirable to further define the characteristics of FBS on cultured cells such as bEC.

We have confirmed that the cytotoxic effect of LPS on bEC is enhanced in the presence of FBS, and that cytotoxicity is dependent on the presence of FBS-related serum factors when LPS is at low concentrations. This observation was previously described by other researchers (12,39), and is now common knowledge among those who have worked with LPS and bEC. The susceptibility of bEC to LPS-mediated cytotoxic effects in vitro appears specific to ruminant animal-derived endothelial cells, because other sources of cells including human, canine, and caprine do not suffer similar cytotoxic effects.
Rather than cytotoxicity, non-ruminant-derived endothelial cells are generally activated by the presence of LPS (8). The bovine serum factors responsible for enhancement of the effect of LPS appear to be complement-independent, and damage to bEC is not inhibitable by a variety of agents (12). More recently, other investigators cultivating bovine endothelial cells have noted that LPS-induced cytotoxicity was enhanced by sera derived from a number of sources, including human, bovine, fetal bovine, equine, porcine, monkey, and rabbit sources (1,8,29), suggesting similar responsible serum component(s).

We have previously demonstrated the existence of LPS-binding factors including LBP in bovine serum (16,17), and surmised that the soluble LPS-binding serum factors may be related to cytotoxicity of bEC. Other investigators have found that the cytotoxic effect produced by LPS with human serum on the bovine endothelial cells was potent at 6.0% (1) or 6.25% serum (8,29) (vol/vol) in cell culture medium. Detectable LPS-related cytotoxic effects were observed at 3% human serum in one study (29), or at 0.5% in another (1). In our current work, we have found that when using LPS and bovine serum on bovine endothelial cells, the evident LPS-mediated cytotoxicity occurs at 0.5% FBS (Figure 4-3), which is in agreement with an earlier report (12). In addition, we found that the LPS-enhancing effects on bEC activation (i.e. tissue factor expression) were apparent at concentrations of FBS or NBS as low as 0.08% vol/vol in the culture medium (Figure 4-1). In general, our work concurs

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with the results of previous investigators, but also indicates that in addition to cytotoxic effects on endothelial cells, the responsible serum components enhance LPS-mediated activation of bEC. Other investigators have recently demonstrated that sCD14 participates in LPS-mediated activation of human endothelial cells, as shown by activation of NF-κβ and increased mRNA encoding tissue factor (33), surface antigen expression (8, 14, 31), and cytokine secretion (31).

In our study, we found that while concentrations of 0.4% to 2.0% FBS were sufficient for amplifying the LPS-mediated effects, the stimulatory effects of FBS actually decreased at high (10%) FBS concentrations. This finding suggests that at high concentrations of FBS, other serum factors may compete for LPS and may result in diminished effects on the bEC. It has been previously indicated by others that in addition to sCD14 in human serum, other serum factors compete for LPS. LBP (37, 44), high density lipoproteins (HDL) (4, 47, 48), anti-LPS antibodies (9, 11, 15), and possibly other serum factors all may participate in binding LPS. Whereas LPS bound to LBP or sCD14 is known to be increased in cell-activating potency (36, 44, 52), LPS bound to innocuous agents such as serum lipoproteins is less potent and does not bind to cell surfaces as effectively (4). The effect of other serum components that compete for LPS, bind it, and reduce its potency may mean that less LPS is available for interaction with sCD14 or LBP. In our study, it is possible that less bEC activation with 10% vol/vol FBS/NBS in the media, as compared to 0.4 -
2% FBS/NBS, is due to greater competition for LPS and decreased amounts of available LPS-sCD14 complexes (or LPS-LBP complexes).

We have also demonstrated that CD14 participates in the LPS-FBS-mediated activation of bEC, because anti-CD14 MAb blocked cell activation as measured by tissue factor expression. The efficacy of the anti-CD14 MAb was specifically dependent on concentrations of FBS. Similarly, we found that anti-CD14 MAb diminished the cytotoxic effect of LPS-FBS on bEC, and that higher concentrations (≥ 5%) of FBS could negate the bEC-sparing effect of the antibody. In previous work, other researchers found that while anti-CD14 MAb was efficacious in blocking the effect of LPS + 6% or 6.25% human serum on bEC, the anti-human CD14 MAb could not block the cytotoxicity of LPS + 10% FBS (1) or with 6.25% FBS (8). Based on these observations, it might be concluded that anti-human CD14 MAbs did not recognize a soluble form of CD14 in bovine serum. Our results obtained using high concentrations of FBS corroborate these findings, but we have also extended experimentation to include low concentrations of FBS. We have found that anti-human CD14 MAb 60bd or 60bca could inhibit both bEC activation and cytotoxicity at low concentrations of bovine serum and LPS, but the effect of the antibody can be overcome at higher levels of serum, presumably due to higher levels of sCD14. The MAb may have higher neutralizing activity with its species-specific ligand (humans) than with material derived from different species (cattle). Nevertheless, we have demonstrated that 40 μg/ml of 60bca completely
blocked bEC cytotoxicity mediated by 10 ng/ml of LPS in the presence of 1% FBS.

To further verify the participation of sCD14 in the bEC response to LPS, we determined the effects of rsCD14 and its combination with bovine LBP on LPS-mediated cytotoxicity. These results clearly demonstrate that rsCD14 is required for LPS-mediated bEC cytotoxicity, and bovine LBP synergizes with rsCD14 in this assay. The action of bovine LBP appears indispensable at low concentrations of LPS. Our observations indicate that both sCD14 and LBP are required for interactions of low concentrations of LPS with endothelial cells, reinforcing the roles of these mediators as cofactors in LPS-mediated vascular events. Our results also show that LPS acts on bovine endothelial cells via an similar pathway as has been documented for human umbilical vein endothelial cells.

Other investigators have previously examined human endothelial cells for the presence of membrane-associated CD14, and have failed to demonstrate its presence using immunohistochemical staining or flow cytometric analysis (3,33). Examination of steady-state CD14 mRNA levels using Northern blot analysis indicated very low levels of mRNA from human endothelial cells (33), and our results using bEC were similar. In contrast to bEC, we have previously demonstrated the presence of a CD14-like receptor on bovine macrophages (51). These results suggest that bEC, like the human endothelial cells, do not express significant numbers of membrane-bound CD14 receptor. This data and
the endothelial cell activation/cytotoxicity data imply that a cell-free pool of CD14, not an endothelial cell pool, mediates LPS-related effects on endothelial cells.

In summary, we have demonstrated the effects of rsCD14 and bovine LBP on LPS-mediated activation and cytotoxicity of bEC. In addition, we have found no evidence for membrane-bound CD14 on the surface of bEC, suggesting that the CD14-like activity present in assays using bovine serum, does in fact reside in the serum. Our findings indicate that low concentrations of LPS emanating from gram-negative bacteria require association with LBP and sCD14 in order to elicit pathophysiologic effects on bovine endothelial cells. Further, investigators utilizing FBS for cell culture should anticipate the likely presence of a bovine sCD14-like homolog in the serum, and should consider any effects that it may have on their experimental results. Future research should define the characteristics and concentration of the bovine sCD14-molecule in serum.

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References


Figures

Figure 4-1. Serum dependent tissue factor expression on LPS-stimulated bovine endothelial cells. Endothelial cells were incubated with 50 ng/ml LPS from *E. coli* 055B:5 and various concentrations of either fetal bovine serum (FBS) or normal adult bovine serum (NBS) for six hours. Increased optical density (O.D.) reflects increased tissue factor expression by the endothelial cells. LPS alone had no effect. Values shown are the means ± 1 S.D., n = 4.
Figure 4-2. The effect of anti-CD14 monoclonal antibodies on tissue factor expression by LPS-stimulated bovine endothelial cells. Monolayers of endothelial cells were exposed to LPS (5 ng/ml, *E. coli* 055:B5) with increasing concentrations of fetal bovine serum for six hours. Anti-CD14 MAb 60bd (20 μg/ml) or an equal amount of an isotype-specific control murine IgG were added in some treatments. Increased optical density reflects increased tissue factor expression by the endothelial cells. Values shown are the means ± 1 S.D., n = 4.
Figure 4-3. The effect of anti-CD14 monoclonal antibodies on cytotoxicity induced by LPS on bovine endothelial cells. A) Monolayers of endothelial cells were exposed to LPS (10 ng/ml, *E. coli* 055:B5) with increasing concentrations of fetal bovine serum for 24 hours. Anti-CD14 MAb 60bca (20 μg/ml) or an equal amount of an irrelevant isotype-matched control murine IgG were added in some treatments. Percent cell death refers to a cytotoxic effect of the LPS on the cultured cells. Values shown are the means ± 1 S.D., n = 4. B) Different concentrations of anti-CD14 MAb 60bca were added to the treatments. After 24 hours of incubation, cytotoxicity was measured by a MTT method.
Figure 4-4. The effect of rsCD14 on LPS-mediated cytotoxicity to bovine endothelial cells. A) rsCD14 from 0.2 to 2.5 μg/ml was added to monolayers of bEC, with or without anti-CD14 MAb 60bca (20 μg/ml), followed by addition of 50 ng/ml LPS. B) 10 ng/ml or 50 ng/ml LPS was added to confluent bEC monolayers with or without 1 μg/ml rsCD14 for 24 hours. rsCD14 and 60 bca were added 30 min prior to addition of LPS. Percent cytotoxicity was determined by MTT assay as described in Material and Methods. Results shown are typical of one of three similar experiments.
Figure 4-5. The combined effect of rsCD14 and bLBP on LPS-mediated cytotoxicity to endothelial cells. A) In the presence of increasing concentrations of rsCD14, 20 ng/ml of bLBP +/- MAb 60bca or murine IgG1 were added to endothelial cells, followed by addition of 10 ng/ml LPS. Note that the effect of individual components without LPS is shown at the left (*), and was essentially background level. A combination of bLBP and rsCD14 plus LPS (▲) induced maximal cytotoxicity. In the presence of bLBP, concentrations of LPS (10 ng/ml) and rsCD14 (100-200 ng/ml) required for cytotoxicity were reduced, compared to LPS and rsCD14 alone (also compare to Figure 4-4B; 50 ng/ml LPS and 1 μg/ml rsCD14). Results shown in Figure 4-5A are typical of one of three similar experiments. B) A silver-stained 10% SDS-PAGE gel shows a sample of the chromatographically-purified bovine LBP used in these experiments, with a molecular weight of 65 kD. Std = molecular weight standards. C) Corresponding photomicrographs are shown of endothelial cell
monolayers which received the experimental plates, final magnification X125. 
A. Control. B. LPS alone. C. Bovine LBP alone. D. LPS + bLBP. E. LPS + 
rsCD14. F. LPS + bLBP + rsCD14. G. LPS + bLBP + rsCD14 + MAb 60bca 
(anti-CD14). H. LPS + bLBP + rsCD14 + isotype-matched control MAb 
(IgG1).

(Figure 4-5C)
Figure 4-6. Autoradiograph of northern blot analysis of cellular RNA from bovine endothelial cells, bovine macrophages, and human monocytes using an anti-human cDNA probe for CD14. The human monocytes exhibit the expected band at 1.5 kb (top left), and the bovine alveolar macrophages also exhibit a weak band of this size (top, center). The bovine macrophages also show an unexpected band at 3.1 kb. mRNA from bovine endothelial cells did not hybridize with the probe (top, right). A photograph of total RNA loaded on the agarose gel is shown at bottom.
PART 5

A DUAL BIOLOGICAL EFFECT OF RECOMBINANT SOLUBLE CD14 ON LIPOPOLYSACCHARIDE-MEDIATED ACTIVATION AND BINDING/UPTAKE OF BOVINE ALVEOLAR MACROPHAGES

Abstract

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Specificity of rsCD14-mediated binding/uptake of LPS
sCD14 inhibited LPS binding/uptake in the presence of serum
A combined effect of rsCD14 and bLBP on LPS binding/uptake

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Figures 5-1 To 5-8
Abstract

The effects of soluble CD14 (sCD14) on the activation of myeloid cells by lipopolysaccharide (LPS) is still controversial. In this study, LPS-mediated activation of bovine alveolar macrophages (bAM) was evaluated with a tissue factor expression assay, and the binding/uptake of LPS to bAM in cell culture medium at 37 °C was determined. Our data indicated that sCD14 was able to moderately increase LPS-mediated activation of bAM and LPS-binding to bAM in the absence of LPS-binding protein (LBP). The enhanced LPS-binding/uptake of bAM by sCD14 was dose-dependent and inhibitable by anti-CD14 monoclonal antibody 60bca. At a high concentration of bovine LPS-binding protein (bLBP), the addition of rsCD14 reduced LPS-binding/uptake by about 20%, compared with bLBP alone. However, at low concentrations of bLBP, rsCD14 and bLBP exerted a combined effect on LPS binding/uptake of bAM. Addition of rsCD14 (4 μg/ml) into 10% fetal bovine serum dramatically reduced LPS-binding/uptake of bAM. These results indicate that sCD14 increases activation and binding of LPS by myeloid cells in the absence of other serum proteins, but significantly decreases LPS binding/uptake in the presence of serum. In addition, anti-CD14 MAb modestly inhibited activation and binding of LPS by bAM in the absence of serum factors, suggesting direct interaction of LPS with mCD14 on bAM. Collectively, these results indicate that a dual effect of CD14 on the interaction of LPS with macrophages exists. The predominant LBP-mCD14 pathway usually masks direct effects of sCD14. A
hypothetical model for LPS-mediated activation of both myeloid and non-myeloid cells is discussed.

**Introduction**

Lipopolysaccharide (LPS) is a glycolipid derived from the outer layer of all gram negative bacteria (1). LPS may initiate signs of sepsis by acting locally on cells at specific organ sites of infection, or it may be absorbed into the bloodstream from the gut, mammary gland, lung, and other sites to act systemically. It has been shown that LPS has very limited direct actions, and acts by induction of proinflammatory cytokines and other mediators which cause the clinical signs and pathological sequelae (2-5). Interaction of LPS with target cells is now recognized to involve LPS-binding protein (LBP) and soluble CD14 (sCD14) in serum, and membrane CD14 (mCD14) on myeloid cells (6,7). In the presence of low concentrations of LPS, LPS first complexes with LBP in serum, and the LPS-LBP complex results in a conformational change in LBP, allowing its association with mCD14 molecules on myeloid cells (8-10), or sCD14 in plasma (11-19). The resultant LPS-LBP-CD14 complex triggers activation of certain cells, including macrophages and neutrophils, where the CD14 is a membrane-bound receptor. sCD14 in plasma has been demonstrated to be responsible for LPS-mediated activation of endothelial or epithelial cells. In this case, sCD14 can bind LPS directly or with catalysis from LBP, and deliver LPS to an uncharacterized signal transducer on these nonmyeloid cells.
While our understanding of the interaction of LPS with serum proteins or membrane proteins has been greatly advanced, the precise mechanism remains to be elucidated in different cell types and possibly in different species. Effect of sCD14 on LPS-dependent activation of mCD14-bearing cells is still controversial. Inconsistent data from different studies (20-22) may result from varying experimental conditions including serum, medium, and temperature at which the assay was performed. However, a single mechanism has been recognized in which the action of both sCD14 and mCD14 is to transfer LPS to an unknown LPS signal transducer(s). In this study, we demonstrate that a single mechanism of CD14-mediated LPS transfer has a dual biological effect on LPS-stimulated activation of bovine macrophages. sCD14 facilitates LPS binding/uptake and activation of macrophages in the absence of the other serum factors, but sCD14 mediates inhibition in the presence of serum. In addition, our results in combination with other well-documented studies may suggest that similar pathways for LPS delivery onto both myeloid and nonmyeloid cells exist although their dominant pathways differ.

**Materials and Methods**

**Alveolar Macrophages**

Bovine alveolar macrophages were collected from Holstein-Friesian cattle by bronchopulmonary lavage as has been described (108). Lavage fluid with cells was filtered through sterile gauze, cells were pelleted by centrifugation,
and were resuspended and washed twice in sterile, pyrogen-free buffer before final resuspension in Dulbecco’s Modified Eagle’s Media (DMEM). The percentage of macrophages determined by Wright-Giemsa stained cytocentrifuge preparation was greater than 90%, and viability determined by trypan blue dye exclusion was also greater than 90%. Protocol for collection of alveolar macrophages was approved by an animal care committee at this institution.

**Tissue Factor Assay**

Tissue factor expression on the macrophages was quantitated with a colorimetric assay (23) based on digestion of substrate S-2222 by activated factor X. The assays were performed in 96-well flat-bottom microplates as previously described by our laboratory (24). 5 x 10^4 macrophages per well in DMEM were exposed to various concentrations of LPS (*E. coli* O55:B5) alone or in combination with quantities of rsCD14, fetal bovine serum (FBS), or bovine LBP purified by a sequential chromatographies (described below). In some assays monoclonal antibodies or isotype-matched MAb of irrelevant specificity (control MAb) were added 20 minutes before addition of the LPS+serum factors. After 6 hr incubation at 37°C, the plates were washed three times with phenol-free Hank’s balanced salt solution (HBSS). The reaction was developed at 37°C for 1 hour following addition of coagulation concentrate (Proplex T) and substrate S-2222. The optical density at a
wavelength of 405 nm was quantitated with a Bio-Tek microplate reader. We found the colorimetric assay for tissue factor to be very consistent with that of the modified prothrombin assay (25).

**LPS binding/uptake assay**

The $^3$H-labeled LPS was aliquoted and stored at -70°C. Fresh aliquots were thawed, vigorously vortexed, and diluted in binding medium that consists of DMEM medium containing 300 μg/ml of bovine serum albumin (BSA) and 0.2 mM EDTA. Macrophages were washed several times with the binding medium. sCD14 and other protein factors were incubated with $^3$H-LPS at room temperature for 15 min before employed to cells. 2.5 million macrophages were incubated in a total volume of 250 μl in a 1.5 ml microcentrifuge tube at 37°C with $^3$H-LPS and/or rsCD14, LBP or serum in DMEM binding medium. The reaction tubes were rotated with a Labquake Shake (Labindustries, Inc., Berkeley, CA). Duration of incubation was 2 hours except for an assay determining the kinetics of binding/uptake. At the end of incubation, the cells were centrifuged at 4°C, and liquid was aspirated. Wash buffer was based on a recipe (15), and slightly modified. One ml of wash buffer I consisting of 0.15 M NaCl, 10 mM HEPES, 3 mM EDTA, and 0.3 BSA mg/ml (pH 7.4) was added at 4°C, and the tube was rotated at 4°C for 10 min. The procedure was employed to detach a small fraction of adherent cells from the tube wall and possibly to lower the background binding. The tube was centrifuged, the liquid
was removed, and then 400 µl wash buffer II containing 1 mM EDTA was added. The cells were suspended and transferred to a new tube. The procedure was done to prevent any influence from tube binding of LPS. The cells were pelleted, and solubilized with 200 µl of 1% Triton X-100 plus 50 mM EDTA. The binding/uptake of LPS to the cells was counted in 5 ml Biosafe II cocktail-filled glass vial with a scintillation counter. All assays were done in triplicate, and the experiments were repeated at least once to confirm the results.

In preliminary experiments, we noticed that the binding affinity and specificity were greatly influenced with different medium. To keep consistence with the cell activation assay, DMEM medium was chosen. LPS carrier protein, BSA, is required to minimize tube binding. In addition, 1% Triton X-100 is better than 2% SDS. The latter resulted in viscous, insoluble material which could not be pipetted.

**Preparation of Bovine LBP**

Pooled bovine serum collected from normal, healthy cows was first fractionated using Bio-Rex 70 cationic exchange chromatography as described for rabbit serum (26) and bovine serum (27). The serum fractions were screened for purity and presence of a 60-65 kD protein using 10% SDS-PAGE with reducing conditions, followed by silver staining. Qualitatively similar fractions were pooled, and the identity of the isolated protein (bLBP) confirmed
by N-terminus sequencing (27) directly from the unblotted fractions eluted from the second chromatography using S-200 sephacryl in a 16 x 100 cm column. The samples were confirmed to contain approximately 90% bLBP without detectable CD14 on silver stained SDS-PAGE. The purified bLBP was lyophilized and stored at -70°C until use in the assay.

Media and Reagents

The recombinant human sCD14 was generously provided by AMGEN, Inc., Thousand Oaks, CA. ³H-LPS (lot #5103A) from List Biological Laboratories, Inc., Campbell, CA, is an Rb chemotype isolated from E. coli K12 LCD25. Cocktail used for β ray counting was Biosafe II from Research Products International Corp. Dulbecco’s Modified Eagle’s Medium (DMEM) was purchased from Whittaker MA Bioproducts, Walkersville, MD. Ethylenediaminetetraacetic acid (EDTA) and HEPES were from GIBCO Laboratories, Grand Island, NY, and fetal bovine serum (FBS) was obtained from Hyclone Laboratories, Inc., Logan, UT. FBS contained 0.6 unit endotoxin/ml and 32 mg protein/ml, according to the manufacturer. Bovine serum albumin and LPS from Escherichia coli serotype O55:B5 were from Sigma Chemical Co., St. Louis, MO. Chromogenic substrate B2-Ile-Glu-Arg-P-nitroanilide (S-2222) was from Kabi Pharmaceuticals, Franklin, OH; a coagulation factor concentrate (Proplex T) was obtained from Travenol Labs, Inc., Glendale, CA. Anti-human CD14 MAb 60bca (IgG1) is from a line
established from the 60bd clone, is similar in function to 60bd, and was purchased from ATCC, Rockville, MD. Antibodies were purified from ascites using an ImmunoPure (G) IgG Purification Kit (Pierce, Rockford, IL) and stored without preservative at -70°C until use. The control isotype-matched MAb from Organon Teknika Corp., Malvern, PA, was mouse IgG1 (Kappa), derived from MOPC 21, a mineral oil-induced plasmacytoma. The antibody has no known hapten or antigen binding activity.

Results

sCD14 Increased LPS-Stimulated Tissue Factor Expression of bAM

It has been shown that sCD14 is required for activation of vascular endothelial cell (EC) by LPS. It is also evident that sCD14 reduces activation (20) or binding (21) of LPS by myeloid cells in the presence of serum. Since sCD14 has an ability to transfer LPS in EC/epithelial cells, we tested the possibility that in the absence of LBP or other serum factors, rsCD14 facilitates LPS-mediated activation of bovine macrophages. A chromogenic assay for LPS-stimulated tissue factor expression in bAM was used with addition of rsCD14 in DMEM medium. OD405 nm was 0.019 ± 0.003 (n = 4) at 10 ng/ml of LPS alone, and tissue factor expression was increased by 13.4 fold at 10 ng/ml of LPS plus 1 µg/ml of rsCD14 (OD405 nm = 0.274 ± 0.032). Based on this preliminary result (figure not shown), we compared the effective strength of purified bovine LBP, rsCD14, and LPS alone on tissue factor
expression by varying LPS concentrations (Figure 5-1). As expected and already well documented, bLBP dramatically enhanced tissue factor expression at $\leq 1$ ng/ml LPS. rsCD14 gave 50% of maximal effective response at 6 ng/ml of LPS (EC50), while EC50 of LPS alone was 100 ng/ml. According to previous study with EC treated by LPS and rsCD14 in our lab and others, the rsCD14-enhanced tissue factor expression seems comparable to rsCD14-enhanced activation of EC in light of both concentrations of LPS and rsCD14 used, although we did not design such an experiment to show the quantitative comparison at this time. The results shown in Figure 5-1 would be further supported by the LPS-binding assay described later.

**Anti-CD14 MAb 60bca Inhibited Tissue Factor Expression**

rsCD14-enhanced tissue factor expression by LPS-stimulated bAM was inhibitable by anti-CD14 MAb 60bca, as showed in Figure 5-2A in which an isotype-matched murine myeloma IgG1 was used as a control. In addition, we found that at high concentrations of LPS at which cells were activated without any protein factors, 60bca, but not control antibody IgG1 also inhibited tissue factor expression by a significant amount ranging from 40-60% (Figure 5-2B). This result was consistent with the data in other publications using THP-1 (28), and CD14-transfected 70Z/3 (29) and CHO cell line (30), providing evidence that LPS is able to directly bind mCD14 on, and activate natural myeloid cells.
Time Course of rsCD14-Enhanced LPS Binding/Uptake to bAM

To keep a similar reaction condition to those used in LPS-stimulated cellular activation such as cytokine release and tissue factor expression in myeloid cells, DMEM medium and 37°C incubation were chosen in the following LPS binding/uptake assay. Unlike LPS-binding assays performed at low temperature (4°C or 10°C) in which maximal binding occurs in minutes, LPS-binding/uptake by bAM reached a plateau at the first hour and then slightly increased with the time. Inconsistent with cellular activation in which rsCD14 mediated a 10-20 fold increase, rsCD14 mediated only a 1-2 fold increase of LPS binding/uptake. However, this result is consistent with the well-known fact that binding/uptake of LPS via non-CD14 molecules such as scavenger and CD18 receptors is not so efficient as CD14-mediated binding/uptake for cellular activation. In the following LPS binding/uptake assays, a 2 hour incubation at 37°C was used.

Specificity of rsCD14-Mediated Binding/Uptake of LPS

sCD14-enhanced binding/uptake of LPS was determined for specificity with a dose-dependent response and antibody inhibition assay. As shown in Figure 5-4, the rsCD14-mediated binding/uptake of LPS by bAM was dose-dependent at 37°C, but not at 4°C. The results also indicated the requirement of physiological temperature for CD14 to transfer LPS. The inhibition of LPS-binding to myeloid cells by rsCD14 at 4°C (22) in the presence of serum was
not significant in the absence of serum.

The enhanced LPS binding/uptake of bAM by rsCD14 was entirely inhibited by a relatively high concentration of anti-CD14 MAb 60bca (40 \( \mu g/ml \)), but not control MAb IgG1 (Figure 5-5). This result is another piece of evidence which, in combination of those seen in the cellular activation assay, suggested that significantly increased LPS-stimulated tissue factor expression may be a specific effect mediated by rsCD14. In addition, consistent with Figure 5-2B, binding/uptake of LPS to bAM in the absence of serum or serum factors was modestly inhibited by anti-CD14 MAb 60bca. In three independent assays, the inhibition varied from 19-32%.

sCD14 Inhibited LPS Binding/Uptake in the Presence of Serum

There have been observations indicating a neutralizing effect of rsCD14 on binding of LPS by myeloid cells at 4°C (21), or LPS-mediated activation in whole blood (20). In order to be comparable with functional assays, effects of sCD14 on LPS binding/uptake in the presence or absence of fetal bovine serum were determined in physiological cell culture medium and at 37°C incubation. Contrary to enhanced LPS uptake/binding in the absence of serum, addition of rsCD14 (4 \( \mu g/ml \)) to serum-containing medium significantly inhibited LPS uptake/binding of bAM at 37°C. This result, although obtained at 37°C incubation, was consistent with those performed at 4°C (21). A dose-response curve indicated that rsCD14-mediated inhibition of LPS uptake/binding was
serum-dependent (Figure 5-6). At 0.1% FBS, no inhibition was observed with addition of rsCD14. Peak uptake/binding of LPS mediated by serum occurred at 10% FBS in our assay. LPS alone was 98 ± 7 cpm; LPS + rsCD14 was 181 ± 12 cpm; 10% FBS plus LPS was 720 ± 58 cpm; and rsCD14 + 10% serum + LPS was 338 ± 27 cpm. The specific inhibition mediated by rsCD14 on LPS binding/uptake at 10% FBS was 74.8% \[1-(338-181)/(720-98)\]. At 80% FBS, which is close to in vivo condition, LPS binding/uptake was significantly reduced without addition of rsCD14, compared with that at 10% FBS. Although 4 μg/ml of rsCD14 still reduced LPS binding/uptake from 369 cpm to 247 cpm at 80% FBS, its effect was not so powerful as that at 10% FBS. The reduced in vitro binding/uptake of LPS at high concentrations of FBS may reflect an in vivo phenomena where a considerable amount of sCD14 present in serum has been able to sequester a portion of LPS from target cells.

A Combined Effect of rsCD14 and bLBP on LPS Binding/Uptake

In three independent assays, a combination of bLBP at 160 ng/ml and rsCD14 (4 μg/ml) reduced binding/uptake of LPS to bAM (about 20%), compared with that of bLBP alone. The result indicates that in the presence of LBP, sCD14 is not required for mCD14-bearing cells. The decrease by addition of rsCD14 to LBP-containing medium might be explained as incomplete transfer of LPS by rsCD14, when a dominant LBP-mCD14 pathway exists.

A dose-response curve with bLBP was tested on LPS binding/uptake
(Figure 5-7). Net increase of LPS binding/uptake by addition of 10 ng/ml bLBP was 124 cpm; of 4 μg/ml rsCD14 was 93 cpm; and of a combination of rsCD14 and bLBP was 357 cpm, which is 1.65 fold of the sum of the above two values. Similar data were obtained from three independent assays with bLBP concentrations from 5-40 ng/ml.

**Discussion**

The function of CD14 in LPS-mediated cell activation was not known until 1990 when Wright et al discovered that anti-CD14 antibodies blocked the production of TNFα from LPS-stimulated human blood cultures and the binding of LPS-erythrocyte complex to macrophages (8). A role for mCD14 has been defined in LPS activation of myeloid cells, while sCD14 has been shown to participate in activation of non-myeloid cell types such as endothelial or epithelial cells that normally do not express mCD14 (6,11,13). However, recent studies have raised a controversial issue about the effect of sCD14 on LPS-dependent activation of cells bearing a glyceryl phosphatidylinositol (GPI)-anchored mCD14. An *in vitro* study revealed that rsCD14 has modest inhibitory effects on LPS-stimulated TNFα production in whole blood (20). Another investigation using fluorescein isothiocyanate (FITC)-labeled LPS and anti-CD14 antibodies demonstrated that the addition of sCD14 to 20% serum is able to reduce LPS binding to human and bovine monocytes at 4°C, with the conclusion that endotoxin-neutralizing capacity of soluble CD14 is a highly
conserved specific function (21). However, a recent study has shown that sCD14 promoted LPS activation of CD14-deficient monocytes from paroxysmal nocturnal hemoglobinuria (PNH) (22). Of more interest in this study is that control normal monocytes showed enhanced response to a concentration of LPS (10 ng/ml) in the presence rsCD14. Conflicting conclusions on the function of sCD14 can be drawn from different experimental conditions.

We have previously demonstrated the expression and function of CD14 in bovine alveolar macrophages (24,31). In this study, we first demonstrated that rsCD14 was able to facilitate LPS-mediated activation of bovine macrophages to produce tissue factor in the absence any other serum factors. The efficiency of rsCD14 in promoting LPS-mediated activation of cells is much lower than that of LBP, but much greater than the activation by LPS alone. The effect of rsCD14 is apparently specific, evidenced by dose-dependent response and anti-CD14 MAb-mediated inhibition of response. To further support these results from functional assays, a series of LPS binding/uptake assays were performed in cell culture medium (DMEM) and at 37°C, the same conditions as used in cell assays. rsCD14 in the absence of other serum protein factors, as expected, enhanced LPS binding/uptake by bovine macrophages. The enhanced binding/uptake by rsCD14 was sCD14 concentration-dependent and inhibitable by use of anti-CD14 MAb. In a recent review, Ulevitch and Tobias proposed three models for CD14-associated signal transduction by LPS (6). In model three, the transducing molecule directly binds LPS in the absence of
mCD14, albeit with low affinity. The affinity for LPS is increased in the presence of mCD14. Since the putative signal transducer is supposed to bind LPS, it is logical for rsCD14 to transfer LPS onto it for activation of myeloid cells in the absence of LBP.

Domination of the LBP-mCD14 route in myeloid cells has been well recognized. Our results also showed that in the presence of LBP, the effect of rsCD14 on LPS binding/uptake of macrophages is masked due to the high efficiency of LBP-mCD14 transfer of LPS, indicating that sCD14 is not important for LPS stimulation of mCD14-bearing cells in the presence of LBP. It is also evident that sCD14 reduced cellular activation with trace amounts of LPS (0.5 ng/ml) in the presence of a sufficient amount of LBP (20). At low concentrations of LBP, however, a combination of LBP and rsCD14 enhanced LPS binding/uptake. It appears that at low concentrations of bLBP at which bLBP-mediated LPS binding is not able to saturate all binding sites on macrophages, effects of both rsCD14 and bLBP may be additive or more likely synergistic.

In contrast to the above effect of rsCD14 in the absence of serum, addition of rsCD14 to FBS-containing medium indeed reduced LPS binding/uptake dramatically. Our results, although obtained in DMEM medium and at 37°C incubation, are consistent with a previous LPS binding assay that was performed at 4°C (21). In addition, a serum concentration-response curve in our study indicates that the endotoxin-neutralizing capacity of rsCD14 is
serum-dependent. These results indicate that rsCD14 has a dual biological effect on myeloid cells *in vitro*: facilitating LPS-mediated activation in the absence of serum and reducing in the presence of serum. The latter can be explained by results of a recent investigation showing that LPS can be transferred to high density lipoprotein (HDL) by sCD14 (32). Thus, the reduced LPS binding/uptake is due to sCD14-mediated transfer of LPS to HDL in the serum, so that LPS-associated with HDL is unavailable for cell activation.

In addition, direct binding of LPS has been detected in the vitamin D$_3$-treated THP-1 cell line by a photoaffinity crosslinking methodology in combination with gel migration (29), and in a human CD14 transfected CHO cell line (30). These cells express a high copy number of mCD14 molecules. A functional assay using vitamin D$_3$-treated cells indicates that anti-CD14 MAb is able to inhibit LPS-stimulated interleukin-8 production in the absence of LBP or serum (28). In our study, we further confirm direct interaction of LPS with mCD14 in isolated, natural macrophages from cattle. In the absence of serum or serum factors, the enhanced sensitivity to LPS stimulation in mCD14-bearing cells is associated with the increase in the amount of CD14 on the cell membrane (28). Thus, the presence or absence of mCD14 may contribute to the difference in sensitivity to LPS between myeloid and non-myeloid cells.

LPS concentration and serum factors are important determinants for results of *in vitro* investigations. It appears that entirely different conclusions can be drawn when employing different experimental conditions. Figure 5-8 is
a "three steps-efficiency" model proposed, which is derived from our data and in consideration of data presented in other publications. In the presence of picogram concentrations of LPS, LBP is required to interact with sCD14 or mCD14 for target cell activation. In addition, a small portion of LPS can be sequestered by sCD14 due to incomplete dissociation of LPS from sCD14-LPS complex, and HDL has been found to play an important role in sequestration of LPS via interaction with sCD14 and LBP (32). At slightly higher nanogram concentrations of LPS, sCD14 may transfer LPS onto cellular surfaces for cell activation. LPS transferred by sCD14 is of high affinity for LPS signal transducers on both myeloid and nonmyeloid cells. And LPS is also able to bind mCD14. At very high concentrations of LPS, LPS may directly bind to the transducer in sufficient quantity to trigger cell activation, e.g. in a serum-free response, which is also inhabitable by anti-CD14 MAb. It appears that the routes ("steps") taken by LPS determine the efficiency of cellular stimulation. LPS transfer and binding via sequential interactions of LPS-LBP, LPS-CD14, and finally LPS-transducer (i.e. "three steps"), has the highest efficiency of stimulation on the target cells. In the absence of LBP (two steps), the efficiency is either moderate (LPS-sCD14 for EC and monocytes) or low (LPS-mCD14 for monocytes). The lowest efficiency is one step, namely the direct binding of LPS to a putative signal transducer. It has been well documented that the dominant route for myeloid cells is LPS-LBP-mCD14-transducer, and for nonmyeloid cells is LPS-LBP-sCD14-transducer. It should be noted that
concentrations of LBP and sCD14, and availability of HDL may greatly vary from different sites within a host. Minor route may become important when local LBP concentration is limited. The three step-efficiency model, we think, logically explains variation of LPS stimulation, differential sensitivity to LPS, and similar LPS transfer routes existing in both myeloid and non-myeloid cells. Since there are no precise mechanisms available for explaining the relationship between the interactions of these protein factors and the efficiency of LPS, conformational change of these proteins and LPS following interaction is presumably responsible for the differential efficiency among different steps.

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References


Figure 5-1. rsCD14 increased LPS-stimulated tissue factor expression. Tissue factor expression on the macrophages was quantitated with a colorimetric assay (see Material and Methods). 5 x 10⁴ macrophages per well in DMEM in 96-well flat-bottom microplates were exposed to various concentrations of LPS (E. coli O55:B5) alone or in combination with purified bLBP (160 ng/ml) or rsCD14 (1 μg/ml). After 6 hr incubation at 37°C, the plates were washed three times with HBSS. The reaction was developed at 37°C for 1 hour following addition of coagulation concentrate (Proplex T) and substrate S-2222. The optical density at a wavelength of 405 nm was quantitated with a Bio-Tak microplate reader. Increased optical density (O.D.) reflects activation of macrophages with increased tissue factor expression by the cells. All treatments were done in quadruplicate, and the experiment was repeated to confirm the results.
Figure 5-2. Anti-CD14 monoclonal antibody, 60bca, inhibited tissue factor expression. A). 5x10^4 macrophages per well in 96-well microplates were treated with anti-CD14 antibody 60bca or isotype-matched antibody IgG1 at 37°C for 30 min, and then exposed to 10 ng/ml of E. coli LPS and a varying concentration of rsCD14. After 6 hour incubation at 37°C, tissue factor was quantitated with a colorimetric assay (see Material and Methods). rsCD14-mediated increase of LPS-stimulated tissue factor expression depended on rsCD14 concentration, and was inhibitable by anti-CD14 antibody 60bca. B). Macrophages were pre-incubated with anti-CD14 antibody 60bca or control antibody IgG1, and then exposed to 10-1000 ng/ml of LPS. Tissue factor expression was measured following 6 hour incubation. 60bca, but not control IgG1, reduced tissue factor expression.
Figure 5-3. Timing course of enhanced LPS binding/uptake of macrophages by rsCD14. Macrophages were washed several times with the binding medium. 4 μg/ml sCD14 was incubated with H³-LPS at room temperature for 15 min before employed to cells. 2.5 million macrophages were incubated in a total volume of 250 μl in a 1.5 ml microcentrifuge tube at 37°C with of H³-LPS (40 ng/ml) or/and 4 μg/ml rsCD14 (in DMEM binding medium, with rotation by a Labquake Shake. At the end of each timing point, the cells were centrifuged at 4°C, and liquid was aspirated. 1 ml washing buffer I was added at 4°C, and the tube was rotated for 10 min. Liquid was removed after centrifugation, and then 400 μl washing buffer II was added. The cells were suspended with washing buffer I, and transferred to a new tube. The cells were pelleted, and solubilized with 200 μl of 1% Triton X-100 plus 50 mM EDTA. The binding of LPS to the cells was counted in 5 ml Biosafe II cocktail-filled glass vial with a scintillation counter. All assays were done in triplicate, and this is one representative of three independent assays.
Figure 5-4. Dose-dependent effect of rsCD14 on LPS binding/uptake of macrophages. Varying concentrations of rsCD14 were mixed with 40 ng/ml LPS, incubated at room temperature for 15 min, and then added into 1.5 ml vials containing 2.5 million macrophages/vial in 0.25 ml DMEM binding buffer. After 2 hour incubation at 37°C, the cells were pelleted at 4°C, incubated with washing buffer II for 10 min at 4°C, transferred to a new tube, pelleted, and finally β-ray radioactivity was counted. The assay was done in triplicate, and the experiment was repeated twice.

Figure 5-5. Anti-CD14 antibody reduced LPS binding/uptake in the presence of rsCD14 and in the absence of serum. Macrophages were incubated with binding medium, 20 μg/ml anti-CD14 60bca or control IgG1 at room temperature for 30 min. H³-LPS was mixed with medium, 60 bca or IgG1, and incubated at room temperature for 15 min. The mixture was added into 1.5 ml tubes with macrophages, the final volume was 0.25 ml with 40 ng/ml H³-LPS, and 40 μg/ml antibody. Following 2 hour incubation at 37°C, the cells were washed (see Material and Methods), and counted for β-ray radioactivity. The experiment was done in triplicate and repeated twice.
Figure 5-6. Decreased LPS binding/uptake of macrophages by rsCD14 is dependent upon serum concentration. Different concentrations of fetal bovine serum in binding medium were mixed with $^3$H-LPS, or/and rsCD14, and incubated at room temperature for 15 min. The mixtures were added in the tubes with macrophages in 0.25 ml DMEM binding medium, and incubated at 37°C for 2 hours. Cells were washed (see Material and Methods), and counted for β ray radioactivity. The experiment was repeated once.

Figure 5-7. Effect of a combination of rsCD14 and bLBP on LPS binding/uptake of macrophages. A series of concentrations of bovine LBP were mixed with $^3$H-LPS (40 ng/ml) or/and rsCD14 (4 µg/ml), and incubated at room temperature for 15 min. The mixtures were added into the tubes with 2.5 million macrophages in 0.25 ml DMEM binding medium, and incubated at 37°C for 2 hours. Cells were washed (see Material and Methods), and counted for β ray radioactivity. The experiment was repeated twice.
Figure 5-8. Three steps-efficiency model for interaction of LPS with putative signal transducer(s) on myeloid and nonmyeloid cells via different routes. See text for details.
Zhengang Yang was born in Zhenping County, Henan Province, The People’s Republic of China on April 9, 1954. He attended primary, middle and high schools in Zhenping county, where he graduated from Jiasong High School in 1973. After teaching at Dawangmiao Primary School and farming for five years, he entered Henan Medical University (China) in 1978 where he received the Bachelor of Medicine degree in July, 1983. Following one year of residency training in epidemiology at Xinxiang Health and Anti-epidemic Center (China), he returned to Henan Medical University to begin a graduate program in medical immunology in 1984 and he received the Master’s degree in medicine in July, 1987. He then taught nutrition and food hygiene as a lecturer for two and a half years at Henan Medical University. In January of 1990, he came to The United States of America as a visiting scholar where he worked at New Jersey Medical School under the direction of Dr. John D. Bogden. He was recruited to the University of Tennessee as a post-doctoral research associate in May, 1991. While a research associate in 1993, he began to pursue a Ph.D. in the Department of Pathology under the supervision of Dr. Philip N. Bochsler. In the Fall of 1995 he received Ph.D in Comparative and Experimental Medicine at the University of Tennessee.