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Identification and characterization of bovine Lipopolysaccharide-binding protein

Lajwanti S. Khemlani

To the Graduate Council:

I am submitting herewith a dissertation written by Lajwanti S. Khemlani entitled "Identification and characterization of bovine Lipopolysaccharide-binding protein." 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.

Philip N. Bochsler, Major Professor

We have read this dissertation and recommend its acceptance:

David Slauson, Albert Ichiki, Roher Carroll

Accepted for the Council:

Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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IDENTIFICATION AND CHARACTERIZATION OF BOVINE LIPOPOLYSACCHARIDE-BINDING PROTEIN

A Dissertation

Presented for the

Doctor of Philosophy

Degree

The University of Tennesse, Knoxville

Lajwanti S. Khemlani

May 1994

DEDICATION

This dissertation is dedicated to my beloved late parents

Mrs. Lachmibai S. Khemlani

and

Mr. Sobhrajmal Chandiram

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ABSTRACT

Lipopolysaccharides (LPSs, endotoxin) are a major component of the outer membrane of gram-negative bacteria. LPS possesses little intrinsic biological activity. However, within the host, it is capable of playing a major role in the production of several potent proinflammatory mediators. These mediators or cytokines in moderate amounts are crucial for host defense. However, excessive production of these mediators can lead to septicemia, which can prove to be fatal. Several recent studies have indicated that the host has developed endogenous regulatory mechanisms in order to deal with bacterial infections. It has also been shown that some of these immunoregulatory mechanisms include proteins which, upon binding to LPS, can either potentiate or attenuate its effects. A 60 kDa LPS-binding protein (LBP) has recently been identified and characterized from rabbit, murine and human serum. LBP binds to LPS with high affinity and the LPS-LBP complex interacts with a membrane bound receptor called CD14. This interaction has been shown to shift the threshold for LPS induced activation of cells such as monocytes and macrophages. Previous studies done with other systems has led to the speculation of the existence of such an analog in cattle. This research project shows the presence of an analogous LPS-binding protein in the bovine serum. Using photoaffinity labeling studies, a 60 kDa LPS-binding protein has been identified in the adult normal bovine serum. Fractionation of this serum using ion-exchange chromatography (Bio-Rex 70 column and HPLC,

Mono Q column) and photoaffinity labeling studies utilizing ^{125}I-ASD-LPS has enabled the identification of a pool or fraction of serum that contains the 60 kDa LPS-binding protein. These studies show that the 60 kDa protein elutes at 220 mM NaCl. NH_2-terminal sequencing of the first 20 amino acids has shown that this 60 kDa protein has significant sequence homology with previously characterized LBPs. Furthermore, tissue factor assays done with bovine alveolar macrophages stimulated with 1 ng/ml LPS in combination with normal bovine serum and bovine serum fractions containing the 60 kDa LPS-binding protein, obtained at two different stages of purification, has shown that the action of LPS is augmented from 100-1600 fold in the presence of bovine serum and the above protein fractions. The stimulatory effect of LPS-LBP complex has been shown to be mediated through the CD14 receptor. This has been shown by including two concentrations of anti-human CD14 monoclonal antibodies (10 and 20 μg/ml) in the biological assays. Heating protein fractions at 56°C, 60°C and 75°C prior to addition to macrophages has shown that this is a heat labile protein. Incubation of polymyxin B (10 μg/ml) with LPS before the tissue factor assay has shown a reduction in the tissue factor expression. This study suggests that the protein fractions used in the assays bind to the lipid A region of LPS.

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FIGURE PAGE

I shall be telling this with a sigh

Somewhere ages and ages hence:

Two roads diverged in a wood, and I-

I took the one less traveled by,

And that has made all the difference.

(The Road Not Taken)

ROBERT FROST

PART 1

GENERAL INTRODUCTION AND OVERVIEW

CHAPTER 1

INTRODUCTION AND OVERVIEW

The management of a serious infection and the systemic response to infection, a syndrome called sepsis, is one of the most frequent and serious problems confronting clinicians. When sepsis results in hypotension and organ dysfunction, it is called septic shock.

According to figures from the Centers for Disease Control in Atlanta septic shock occurs at a rate of 175 per 100,000 people yearly (49). This number rises to about 500 per 100,000 for patients admitted to hospitals (49). Of these as many as 25 to 40% die (81). Septic shock is the most common cause of death in intensive care units, and it is the 13th most common cause of death in the United States (62).

Septic shock usually begins with tremor, fever, falling blood pressure, rapid breathing and heart beat, and skin lesions (49, 81). It can progress rapidly, within days or hours, and leads to spontaneous clotting in the blood vessels, severe hypotension, and multiple organ damage, mostly due to circulatory collapse and oxygen-free-radical damage (49, 81). Sepsis is an example of a systemic inflammatory response that can be triggered not only by infections but also by noninfectious disorders, such as trauma and other disorders such as pancreatitis (81).

The pathogenesis of septic shock can be initiated not only by endotoxin-containing gram-negative organisms, but also by gram-positive organisms and fungi (81). The pathogenic cascade due to these infections begins with the proliferation of microorganisms at the site of infection (81). The organisms may invade the host in more than one way. They may either enter the bloodstream directly (leading to positive blood cultures) or may proliferate locally and release various substances such as endotoxin and exotoxin into the bloodstream (81, 88). Endotoxin is a structural component of the microorganism, whereas exotoxins are synthesized and released by bacteria into the extracellular environment (81, 88). Although any bacterial infection, in the host, can lead to septic shock, gram-negative bacterium in particular evoke it (49, 81). It is now generally agreed that the clinical problems associated with gram-negative sepsis occur as a result of the host response to endotoxin (lipopolysaccharide; LPS), a major component of the outer membrane of all gram-negative bacteria (84, 85, 88).

LPS by itself possesses little intrinsic biologic activity, however, within the host, LPS, in low concentrations, typically a few μg/L, has been shown to incite complex immune reactions which on one hand are beneficial to the host, but, on the other hand, when uncontrolled can lead to much of the damage associated with septicimia (49, 87, 88). Bacterial endotoxins as discussed by Rietschel and Brade (88) are "molecules that are at once brutal and beneficial to humans. They possess an intrinsic fascination that is nothing less than fabulous. They seem to

have been endowed by nature with virtues and vices in the exact and glamorous proportions needed to render them irresistable...."

Once within the blood stream, endotoxins recruit particular host cells to do their function, that is, induce cells such as monocytes or macrophages, endothelial cells, neutrophils and others to secrete endogenous mediators (8, 49, 78, 80, 81, 85, 88). These mediators act locally or float through the blood, or both, independently, together or in sequence to elicit a myriad of responses (49, 81, 85, 88). Endotoxin stimulated macrophages have been shown to produce proteins such as tumor necrosis factor (TNF-α), interleukin-1 (IL-1), IL-6 and IL-8; oxygen free radicals such as oxygen (O_2^-), hydrogen peroxide (H_2O_2) and nitric oxide (NO) (39, 49, 85, 94, 128). Activated macrophages also release lipids such as prostaglandin E_2 (PGE_2), thromboxane A_2 (TXA_2) and platelet-activating factor (PAF) (49, 85, 94). The production of these mediators regulate the further production of more endogenous mediators with complex effects. It has been shown that TNF-α amplifies the immune response, whereas PGE_2, for instance, negatively regulates further induction of mediators (88, 107).

In low levels the above mediators have beneficial effects on the host. Moderate fever, generalized activation of the immune system and microbial killing can help eradicate the immediate infection. However, when there is an excessive production the host suffers harmful effects such as high fever, hypotension, disseminated blood clotting and lethal shock which can ultimately lead to death (49, 81, 85). Lethal effects occur when bacteria themselves gain entry into the

blood, multiply rapidly in the medium, and in the process, liberate an excess of toxin to further stimulate immune system cells such as macrophages (38, 62, 69).

Thus, a paradox exists in this host/pathogen interaction which has been most aptly described by Lewis Thomas in his book *The Lives of a Cell :*

> "Our arsenals for fighting off bacteria are so powerful, and involve so many different defense mechanisms, that we are more in danger from them than from the invaders. We live in the midst of explosive devices; we are mined....When we sense lipopolysaccharide, we are likely to turn on every defense at our disposal....There is nothing intrinsically poisonous about endotoxin, but it must look awful, or feel awful, when sensed by cells....Endotoxins are read by our tissues as the very worst of bad news....we are likely to turn on every defense at our disposal; we will bomb, defoliate, blockade, seal off, all our tissues in the area...All of this seems unnecessary, panic-driven (49, 63)."

LPS can be released during active growth of gram-negative bacteria, as well as by lysis of the organisms (84, 88). As such antibiotics, given by clinicians, to eradicate bacteria during sepsis syndrome initially worsen shock caused by gram-negative septecemia because they kill the bacteria however, the dying organisms release even more endotoxin into the body.

It is very difficult to maintain an endotoxin free environment, because endotoxin is ubiquotous. Within a host, the source of endotoxin is the gut, with the oral cavity and the lung being other potential sources. Airborne and other sources of endotoxin may induce an inflammatory reaction in the lung (11, 49, 81). Profound proinflammatory changes in the alveolar macrophages, capillary endothelium, platelets, and neutrophils may occur. Activated cells release a myriad of cytokines that could eventually contribute to septic shock: chemotaxis and activation of leukocytes, production of adhesion molecules by endothelial cells

resulting in increased vascular permeability (6, 49, 81, 82, 88, 124). Macrophages in tissue become activated, cytokines released increase the expression of some cell surface receptors that make these cells even more sensitive to LPS (6, 61, 69, 116, 121) and the cyclic phenomenon continues. Activated neutrophils have been shown to release granules containing tissue-damaging proteases and free radicals (49, 63, 81, 88, 94). Changes in the endothelium due to endotoxemia causes release of secondary cytokines such as platelet activating factor which in turn leads to platelet aggregation and fibrin attachment in blood vessels (9). When regulation of normal processes is disrupted, these changes cause widespread coagulation and circulatory collapse, leading to further free-radical damage. All this eventually results in profound hypotension and tissue damage and death ensues within hours.

Once again, it is important to remember that the synthesis of these mediators is crucial for the host defense. Pathogenesis occurs when there is an uncontrolled and exaggerated action of these mediators. Thus, some scientists (63) have aptly referred to these biological mediators as a "two edged sword."

Endotoxin: Structure and Chemical Nature

Gram-negative bacteria have a trilaminar cell wall composed of an inner layer, a middle peptidoglycan layer, and an outer layer (84, 88). Bacterial lipopolysaccharides constitute an integral component of the outer membrane of gram-negative bacteria (Fig 1(A)). LPS is a component of the outer leaflet of the

outer layer of gram-negative bacteria (84, 88). This intimate association of LPS with the gram-negative bacterium cell wall confers upon it the term endotoxin. Thus the two are synonymous. LPS is toxic for most mammals and vertebrates and is the source of the surface O-antigens of the gram-negative bacteria (84, 88).

During the development of a method to isolate endotoxins from a variety of gram-negative bacteria it was found that endotoxins mainly consisted of fatty acids, phosphorous and carbohydrates (84, 88). Thus, lipopolysaccharides are macromolecules composed of, as the name suggests, lipids and polysaccharides. A schematic representation of the chemical structure of LPS is seen in Fig. 1 (B). The macromolecule, LPS, is composed of three distinct regions; a minimally variable glycolipid backbone termed as the lipid A portion, a not so variable, small polysaccharide core, and frequently a highly variable polysaccharide chain, referred to as the O-specific polysaccharide region (84, 88). This region consists of identical, repeating oligoccharide units and extends out from the gram-negative membrane surface. There is a great diversity in the structure of O-chains, and each serotype within a group of gram-negative bacteria is characterized by a unique O-specific chain structure. An O-chain region, typically consists of 20 to 40 repeating units that include up to eight sugars. The sequence and type of sugars within the basic unit and the number of the repeating units often varies (84, 88).

LPS exists as a bilayer of vesicular aggregates in solution because the prototype monomer of LPS is an amphiphile. The amphiphatic nature of LPS

promotes the formation of intermolecular ionic linkages which may lead to aggregation of LPS molecules. Because of this, the molecular weight of LPS isolates varies anywhere from 20,000 or less, to relatively massive aggregates of up to 1 μm diameter (84, 88). The degree of aggregation is reversible. Aggregation of LPS may affect the biologic activity of LPS in an unpredictable manner. Some biologic activities that increase with aggregation are erythrocyte affinity, blood clearance, and lethal toxicity in rats. Other biologic activities may decrease, such as pyrogenicity and lethal toxicity for mice (84, 88). Mitogenecity for B lymphocytes and Limulus amoebocyte lysate gelation (a diagnostic indicator of LPS presence) do not change with aggregation (84, 88).

Different regions of the LPS molecule possess distinct functions. Of these regions, the O-specific side chain provides LPS with a hydrophilic region and since it is exposed to the environment this region also confers serologic specificity (83, 84, 88). The polysaccharide portion of LPS is important for the solubility of LPS thus the pathogenicity of gram-negative bacteria (84). Since, the O-specific region is exposed on the surface of the gram-negative bacterium, it is this region that evokes a specific immune response, that is the of production of specific antibodies. These antibodies recognize only that particular O-chain but no other molecules. Hence, for example, each variant of *Salmonella* will possess a distinctive O-chain and elicit production of different antibodies (17, 84, 88).

The core region of LPS links the lipid A region to the O-specific region (84, 88). In response to mutant endotoxins that lack an O-chain, the core region

can trigger antibody production. The core region of LPS is divided into two regions the inner and the outer core (84, 85, 88). It is the inner core that is linked to the lipid A region, whereas, the outer core is linked to the O-specific region (84, 85, 88). Of the two regions, the inner core consists of unusual sugars such as heptose, which contains seven instead of the usual six carbon atoms (88). The other is a unique sugar called Kdo (3-deoxy-D-manno-2-octulosonic acid) (88). Kdo consists of eight carbon atoms and occurs nowhere else in nature, except in certain plants and algae. It is present in all endotoxins and links the polysaccharide to the lipid. The uniqueness of this region makes it a potential target of therapy. Thus, both the above regions of LPS play an important role in the action of LPS. However, it is the lipid A region that has been known to be responsible for most of the actions of LPS (36, 43, 84, 88, 106).

Several studies have revealed that the lipid A and the core region tend to remain structurally similar among all gram-negative bacteria. Thus they are considered to be the most conserved regions within groups of bacteria; e.g. similar for most *Salmonella species* (84, 85, 88). Westphal and Luderitz, in 1954, found that lipid A consisted mainly of a sugar, glucosamine, as well as phosphate and long chains of fatty acids, each with a backbone of about 14 carbon atoms (84, 85, 88). For example, in *Salmonella minnesota,* the lipid A region consists of diglucosamines, each of which is phosphorylated (65, 66, 69). The importance of each of these molecules in the lipid A region has been shown recently in studies

whereby the removal of the phosphate groups or the long chain of fatty acids renders the lipid A region or the endotoxin inactive (23, 41, 84, 85, 88).

Based on the colonial morphology, some bacteria are termed rough (R) mutants (9, 84, 88). In this group, there is an incomplete side chain synthesis and consequently an exposed core determinant. There are two main groups of R-mutants, Ra and Rb - Re mutants (9, 84, 88). Ra mutants are defective in the O chain biosynthesis and therefore have only the lipid A region and the core region. Rb - Re mutants are defective in core biosynthesis, therefore produce a LPS molecule composed a fragment of the core linked to the lipid A. R mutants have been isolated from *Salmonella, E. coli, Shigella, Proteus and others* (9, 84, 88).

Studies have shown that the highly conserved lipid A moiety of LPS possesses the biologic activity and there is now increasing evidence that it is this region that possesses the vices and virtues of endotoxin (43, 84, 85, 88, 106). Thus, the lipid A region not only causes the ill effects of endotoxin, but is also responsible for enhancement of immunity (88, 106).

Acute Phase Response Proteins

Tissue injury, trauma or inflammatory stimuli alter a large number of normal homeostatic mechanisms (47, 55, 94). In the blood these alterations from the normally operative mechanisms constitute the acute phase response (47, 55, 95). In experimental animals this response can be induced by injection of turpentine or LPS (55, 98). This stimulus, within the first few hours, results in

changes in pleiotropic cellular processes that are normal responses to injury, but could be potentially lethal.

The ability of LPS to incite extraordinary proinflammatory changes in the host's own immune system has been well documented (39, 49, 51, 63, 95). Some of these changes that occur due to acute phase response involve a change in the concentration of some plasma proteins, called acute phase proteins (47, 56). In general, in all mammalian species studied there is an increase in proteins such as α1-acid glycoprotein, fibrinogen and haptoglobulin while there is an accompanying decrease in the others such as albumin (47, 55). Typically, the site for the synthesis of most acute phase proteins is the liver, specifically hepatocytes (47, 55).

The extent of the acute phase response is related to the severity of the inflammatory state or the extent of tissue injury. To date several acute phase reactants have been identified and biochemically characterized (47, 55, 89, 91, 96, 98). However, the functions of all these proteins have not been identified. Results show that most of these proteins vary considerably in their physicochemical character and known functional capabilities (47, 55, 105). Thus, even though the presence of similar proteins has been identified in different species, variablity has been shown to exist even between closely related species such as mouse and rat. In humans both C-reactive proteins (CRP) and serum amyloid A protein (SAA) are major acute phase proteins, while neither serum amyloid P protien (SAP) or α_2-macroglobulin manifests acute-phase characteristics (47, 55). In mouse, both CRP and SAA are present, with CRP levels being much

lower to those in humans (47, 55). In rats, however, SAA is totally absent with CRP levels being comparable to those in mice (47, 55). SAP is present only in mice and not in humans and rats.

The state of acute phase response can be transient and reverts to normal if the stimulus is degraded or the host is treated successfully (50, 51, 55). However, if inflammatory disease persists it could result in a chronic response and this could potentially lead to cachexia. Thus, it is not surprising that just like everything else in nature, the host has been endowed with means and mechanisms, both, to support (eg. *E. coli* in the gut) and to combat, if needed, the unwanted assault, so to speak, of invaders such bacteria.

Regulatory mechanisms have been evolved by the host in order to respond to bacterial infection and combat septicemia caused by gram-negative bacterial infections. One such mechanism involves the presence of antimicrobial peptides in the granules of leukocytes. Recent studies have identified and characterized, to a certain extent, antimicrobial poplypeptides called Bac5, Bac7, bactenecins and CAP18 from neutrophils (59, 67, 125) and murine microbicidal proteins (MUMPs) from murine macrophages (48).

Endotoxins from sources such as the gut, the oral cavity and the lung is transported to the portal circulation and to the liver (50, 72). In a normally functioning liver, LPS is substantially cleared from circulation. This is rapidly accomplished by the liver. At a slower rate the same role is also played predominantly by high-density lipoproteins (HDLs) (75, 97, 104, 105). It has been

suggested that HDL substantially reduce endotoxicity by masking the lipid A moiety in the phospholipids of lipoproteins (85, 104-106).

Several other studies have shown the existence of other regulatory mechanisms whereby the host responds to LPS by clearing LPS from circulation and by "partitioning" it in the plasma (95, 97-100, 102, 106, 109). Recently in the process of studying these mechanisms in normal and acute phase mammals several other proteins have been identified that have been shown to play an important role in the bioavailability and modulation of LPS action.

Scientists, in the past few years, have shown the presence of a group or a family of proteins that bind LPS and either potentiate, or attenuate, the action of LPS. Some of these proteins function as cell-surface LPS-receptors and others as serum LPS-binding proteins (14, 36, 64-66, 77, 102, 106, 120). Of these LPS-binding proteins, the one referred to as LBP, initially identified and purified by Tobias et. al. (98, 102) from rabbit serum has been well characterized. More recent studies have shown the presence of LBP in human (89-91, 102, 106) and murine (31, 102, 106) serum. The other most recently characterized protein from the rabbit, human and mouse system is CD14 (90, 91, 102, 106, 118).

Wright et. al. (119) have identified and characterized yet another novel factor in plasma called septin that binds LPS (122). In addition, bactericidal/permiability-increasing protein (BPI) from human neutrophils is another well studied LPS-binding protein (19, 22, 32, 76, 79, 114). Acyloxyacyl hydrolase (AOAH) (23, 39, 85), scavenger receptor (41, 85), also referred to as the

acetyl-low-density-lipoprotein receptor, CD18 molecules (leukocyte integrins) (85, 120), the 73 kDa LPS receptor on murine splenocytes (64, 66), the 18 kDa LPS receptor on the murine macrophage-like 7OZ/3 cell line and the 55-65 kDa LPS receptor on the J774.1 murine macrophage-like cell line are some other proteins that are involved in interactions with LPS (120, 106).

Briefly mentioned below are the salient features of some of the above mentioned LPS-binding proteins that have been evolved by the host in an attempt to modulate the availability and action of LPS (Fig. 2).

LBP, Septin and CD14: During the past several years, studies done by Ulevitch, Tobias, Schumann and their co-workers (73, 74, 89, 90, 91, 96-106) and Wright et. al. (118-120) have unequivocally shown that LBP, in serum, binds both the rough and smooth form of LPS with high affinity (10^{-9}M) and specificity. The LPS-LBP complex has been shown to bind to a glycosylphosphatidyl-inositol (GPI)-anchored plasma membrane protein called mCD14 (Fig. 3). And, this interaction has been shown to enhance LPS effects such that cells become exquisitely sensitive to very low concentrations (nanogram/ml to picogram/ml) of LPS.

Using pulse-chase studies (86), it was shown that LBP is synthesized by hepatocytes as a 55 kDa polypeptide and released into the serum as a 60 kDa glycosylated protein. LBP was discovered as a component of rabbit acute phase serum while studying the interaction of LPS with HDL(s) (97). LBP was shown to be capable of regulating the binding of LPS to HDL in normal rabbit and acute

phase serum (104, 105). In acute phase serum, using isopycnic equilibrium density gradient centrifugation, it was shown that ^{3}H-LPS bound to HDL at a much slower rate compared to HDL(s) in normal serum (98). This was found to be due to the formation of a stable complex between LPS and protein(s) in acute phase serum. The presence of this complex was also noted in the acute phase serum of humans and other laboratory animals such as rats and mice (98, 102, 106).

Fractionation of acute phase rabbit serum using column chromatography revealed that a 60 kDa glycoprotein was responsible for complexing with LPS. Previously, LBP was considered to be present only in acute phase serum, however, upon the recent availability of monoclonal antibodies (mAbs) against LBP, researchers have shown that LBP is also a component of normal serum. In normal human serum, LBP is present at a concentration of 3-10 ug/ml (98, 102, 106). During acute phase response this level goes up to 300 ug/ml (102, 106). In rabbit serum, LBP levels have been demonstrated to increase from 0.5 ug/ml to 500 ug/ml (102, 106). The role of LBP in the enhancement of LPS action has been demonstrated in studies where LBP has been either immunodepleted or repleted in a reaction mixure (102).

Experimental evidence for the role of CD14 as a receptor for LPS-LBP complex comes from experiments done by Wright et. al. (118, 119). These researchers using LPS-coated erythrocytes (E-LPS) and macrophages have shown that LBP functions as an opsonin, allowing binding of LPS to marophages at levels of LPS that are not detected in the absence of LBP. Using a panel of monoclonal

antibodies (mAbs) to human macrophages, binding of E-LPS to these cells in the presence of LBP was inhibited by mAbs to CD14 and not by mAb to other macrophage surface markers (91, 118, 119). CD14 is capable of binding LPS in the absence of LBP, however, in the absence of LBP, much higher concentrations of LPS are needed for cellular activation via CD14 (91, 118, 119). LBP, on the other hand has been shown to interact with CD14, only in the presence of LPS (91, 102, 106, 118, 119).

The function of LPS-LBP complex binding to CD14 and activation of macrophages was demonstrated, once again, by using mAbs. Studies have shown that only mAb to CD14 had the ability to inhibit LPS-induced TNF synthesis in whole blood (102, 106, 118, 119). These studies have suggested strong evidence for the role of mCD14 as a receptor for LPS-LBP.

Several recent studies (28, 52, 102, 106, 112) have further reinforced the view that CD14 endows cells with an extremely sensitive receptor system for LPS-LBP complex. Researchers have shown that transfection of cells such as murine 7OZ/3 B cells with the human CD14 gene greatly enhances the responsiveness of these cells to LPS in the presence of LBP, such that LPS-induced cells upregulate surface IgM expression at 1000-fold lower doses of LPS (102, 106, 127). Increased sensitivity to LPS in the presence of LBP was also observed in CHO cells transfected in parallel with the 7OZ/3 cells. Fluorescein-labeled LPS binding to these cells in the presence and absence of LBP supported the above observations (102, 106).

More evidence for the role of CD14 functioning as a receptor for the LPS-LBP complex has been obtained by other set of experiments in which cell lines that do not constitutively express CD14 have been used. A myeloid cell line called THP-1 (15, 106, 127), and the earliest myelomonocytic stem cells represented by HL60 cell line and monoblastic cells represented by U937 have been used for these studies (106, 127). These studies have shown that in these cells induction of CD14 can be stimulated by various agents such as dihydroxyvitamin D_3phorbol esters and gamma-interferon (IFN-γ). Once again, increased LBP-dependent fluorescein-labeled or radiolabeled LPS binding was observed in these cells. Furthermore, when when both CD14(+)-THP-1 and CD14(+)-7OZ/3 cells were treated with a phosphotidylinositol (PI)-specific phospholipase C to remove CD14 (44) or when anti-CD14 antibodies were used, LBP-dependent responses to LPS were significantly reduced and LPB-dependent binding of labeled LPS was abolished.

CD14 has been identified and characterized as a myeloid marker. However, anti-CD14 mAbs have also been shown to react, to some extent, with other cells such as B cells and mammary cells (106, 127). Also, although anti-CD14 mAbs preferentially stain monocytes, reactivity on other cells such as granulocytes has been observed. Thus, it came as no surprise when recent studies identified and biochemically characterized CD14 on neutrophils (45, 106).

Tissue staining studies have shown the presence of CD14 on various members of the monocyte-macrophage lineage, including spleen macrophages,

Kupffer cells, and epitheloid and giant cells in granulocytes (127). These and other studies have also shown that even though macrophages have been shown to express CD14 to a greater extent than other cells, a differential expression of CD14 among these cells exist (127). This has been seen in tissue selections; for instance, where microgliae cells have been shown to be essentially CD14 negative, whereas the perivascular macrophages in the brain have been shown to be CD14 positive. Researchers have shown that peritoneal and pleural macrophages to express higher levels of CD14 as compared to the alveolar macrophages and microgliae cells (127). The differential expression, or dichotomy, of CD14 in tissue macrophges is also observed in cells at different stages of maturation (35, 125). Thus, CD14 has been shown to be absent from early progenitor cells and increases towards maturation towards monocytes (127). In blood, the expression of CD14 depends upon the stimulus and the quality and quantity of stimulus present (18, 56-58, 62). Furthermore, under trauma and pathological conditions, expression of CD14 has been shown to vary (21, 54).

The CD14 gene has been located on chromosome 5 in a region encoding growth factors and growth factor receptors (27). It is this "critical" region that has been shown to be frequently deleted in certain myeloid leukemias (27). Amino acid analysis of CD14 from lapine, human and murine (27, 102, 106) system have shown that CD14 share a "leucine rich motif" with several other membrane bound proteins (102, 106).

Researchers (44, 68, 91, 93) have shown that CD14, a 53-55 kDa differentiation antigen, is present not only on monocytes and macrophages and activated granulocytes, but it also exists as a soluble protein. Soluble CD14 (sCD14) was initially detected in cellular supernants and normal plasma, as a 50 kDa doublet, by its ability to block staining of monocytes using anti-CD14 mAbs (4, 38, 92). Using enzyme linked immunoabsorbant assays (ELISA), sCD14 has also been detected in urine (38). Recent studies have shown membrane CD14 to be shed upon activation with LPS and TNF-α (5, 105). Thus, these treatments increase the presence of sCD14 whereas cytokines such as IFN-γ or IL-4 decreases the sCD14 levels in cellular supernants. Treatment of cells with both phosphoinositol-phospholipase C or proteases has also shown to increase the level of sCD14. However, a different molecular weight species of sCD14 is released by these treatments. sCD14 released via the action of PI-specific phospholipase migrates on a SDS-PAGE as a doublet in the 50 kDa range, whereas the sCD14 obtained due to the protease treatment migrates as a single band. Thus, it is obvious that the PI-specific lipase and proteases cleave mCD14 at different sites.

Levels of sCD14 have been shown to increase in severely burned and polytraumatized patients (54). Increased levels of sCD14 and thus decreased levels of membrane associated CD14 have also been found in a disorder such as paroxysmal-nocturnal hemoglobulinuria (PNH), a disease characterized by a defect in the GPI-anchoring mechanism (21). Cells from these patients exhibit decreased

functional responses to LPS, but the consequences of this decreased responsiveness are currently unknown.

Several researchers have demonstrated the ability of sCD14 to bind both LPS and LPS-LBP complex (3, 46, 83). Initially the role of sCD14 was suggested to neutralize endotoxin (92). However, other studies have recently shown that uncontrolled activation of cells, thus septicemia develops in patients inspite of the increased levels of sCD14. Therefore, a role for sCD14 other than neutralizing endotoxin has been put forth by several other researchers (3, 46, 83, 106, 108). Support for this hypothesis has recently been demonstrated by studies where the addition of serum fractions containing sCD14 and recombinant sCD14 (rsCD14) (46) has been shown to activate cells in the presence of LPS-LPB complex. Once again, cells have been shown to be activated by very low levels of LPS in the presence of LBP. Thus these researchers have hypothesized that sCD14 functions more as a co-ligand in the cellular activation by LPS-LBP complex (46, 83, 106, 107) rather than a "sink" for LPS.

Recent studies done to identify signals that follow binding of LPS-LBP complex to CD14 have provided evidence that LPS activates macrophages via tyrosine kinase activation (33, 113, 123). These researchers have also shown that LPS-induced tyrosine phosphorylation is mediated by mCD14 (33, 113) even though these receptors are known to lack an intracellular cytoplasmic domain which is characteristic of most membrane bound receptors. This has led researchers to suggest the possibility that perhaps mCD14 is closely associated

with another protein which could in fact the "true or real" LPS-receptor. Perhaps this protein could be the one actually involved with the transmembrane signal transduction (70, 106). The role of mCD14 then could be to bring LPS into close proximity to this "other" LPS-receptor. The other hypothesis that has been put forth by this group of researchers (106) is that perhaps mCD14 is part of a receptor complex.

The possibility that sCD14 has functions other than endotoxin-neutralization comes from studies in which endothelial and epithelial cells were activated by LPS in presence of LBP and sCD14 contaning serum. Endothelial and epithelial cells have been shown to be essentially CD14 negative. These and other studies have now lead researchers to believe that sCD14 functions as a co-ligand, such that it facilitates the cellular recognition of LPS (46, 106). In the presence of LBP, once again, this occurs at lower LPS concentrations (46, 106).

All the above studies provide clear evidence for the significance of the LBP and CD14 in the modulation and transduction of LPS action. It has also been unequivocally demonstrated that LBP plays a crucial role in immunoregulating LPS action in the host by making the host exquisitely sensitive to the presence of minute levels of LPS.

Septin is an LPS-binding activity that has been recently identified and characterized from human serum by Wright et. al. (122). This group has shown that septin can opsonize LPS and can be obtained only when at least two protein

fractions are combined. Furthermore, in order for this protein(s) to perform its function, that is, LPS-opsonization, the presence of proteases is needed.

The same researchers using erythrocyte labeled LPS and anti-CD14 monoclonal antibodies have also shown that CD14 mediates binding of plasma-opsonized LPS to macrophages (122). Because of the presence of an enormous amount of septin in normal hosts, septin has been proposed to detect LPS concentrations of lower than 0.1 ng/ml and play a major role in its opsoniztion in normal hosts.

Bactericidal/permeability-increasing protein: BPI is a 55-60 kDa cationic LPS-binding protein present in the azurophilic granules of polymorhonuclear leukocytes (19, 22, 32, 76, 79, 114). Studies have shown that it is a potent cytotoxin and has a remarkable ability to specifically neutralize the activities of only gram-negative bacteria and not fungi or mammalian cells (99). Cloning of cDNAs, for LBP and BPI, and elucidation of the complete amino acid sequence has shown that a 45% overall sequence homology between LBP and BPI exists (99, 106). Both of these proteins have also been shown to be related to the plasma protein, cholesteryl ester transfer protein (CETP) (85).

Further characterization of this protein, has identified the region of BPI that contains the anti-bactericidal activity. Ooi et. al. (79) have shown that a proteolytic fragment of approximately 25 kDa, corresponding to the N-terminal half of human BPI, possesses most of the antimicrobial activities exhibited by the

55 kDa holoprotein. Support for the above evidence comes from most recent studies in which recombinant NH_2-terminal fragment of BPI (76, 114) has been utilized. These studies have shown that rBPI fragment can not only cause killing of serum-resistant gram-negative bacteria in whole blood and inhibit LPS-induced tumor necrosis factor (114), but, it can also inhibit induction of leukocyte responses by LPS (76). Both BPI and LBP have been shown to bind lipid A region of LPS. However, the biologic properties of these proteins have been shown to differ; and this is reflected in the fact that BPI does not amplify LPS-triggered host responses whereas LBP does.

CD18: CD18 molecules also known as leukocyte integrins or β2 integrins comprise of a family of several closely related, dimeric cell-surface glycoproteins (85). These antigens present on macrophages bind particulate LPS and deliver it to a degradative compartment without influencing the induction of cytokines (94). Thus, it has been suggested that CD18 antigens participate in direct clearance of LPS from circulation without participating in the secretory responses related to LPS; a role which has been shown to be played by CD14 molecules. Anti-CD18 mAbs have been shown to reduce tissue damage in animal models of infection and tissue ischemia, but this has been suggested to be likely due to direct inhibition of neutrophil adherence to endothelium rather than anti-endotoxin action (85).

Scavenger Receptor : The scavenger receptor, also known as the acetyl low-density lipoprotein (LDL) receptor, is a 95 kDa trimeric transmembrane glycoprotein that recognizes several ligands (41, 85). Raetz and colleagues (85) using a bioactive radiolabeled lipid A precursor [^{32}P]lipid IVa have shown saturable binding to the surface of the murine macrophage-like cell line, RAW 264.7. The scavenger receptor has been shown to bind other ligands such as acetylated LDL and polyinosinic acid. The scavenger receptor has been cloned and evidence for lipid IVa as being the ligand for the scavenger receptor comes from transfection of CHO fibroblasts with cloned scavenger receptors. Acetylated-LDL and other ligands of this receptor competitively block the binding of [^{32}P] lipid IVa, and vice versa.

Binding to the scavenger receptor facilitates the entry and subsequent dephosphorylation of lipid IVa at 1-position (85). The strategic location of this receptor in liver, specifically in Kupffer cells and sinusoidal endothelials cells plays a pivotal role in removing endotoxin entering circulation from the gut. These receptors have been shown to pinocytosize LPS and digest it (85). Evidence for this arrives from studies in which chloroquine has been present in the medium. Chloroquine is known to block acidification of endosomes. Presence of this reagent in these studies have shown to block degradation of LPS.

The pinocytosis of LPS by the scavenger receptor does not result in the induction and secretion of cytokine production. This has been suggested from experimental studies that have shown that even though acetylated LDL completely

blocks [^{32}P]lipid IVa binding to scavenger receptor it does not block lipid IVa induced cell activation (85). Furthermore, cell activation by lipid IVa which has previously been shown to mimic LPS activity, does not alter LPS-induced cytokine production (85).

Dephosphorylation of both lipid IVa and lipid A has been shown to be associated with reduced toxicity (85). Therefore, it has been suggested that the scavenger receptor may have biological relevance because of its role in the detoxification of LPS *in vivo.*

Acyloxyacyl Hydolase (AOAH) : AOAH has been identified in humans and in rabbits (23, 40, 67, 85). Erwin and Munford have shown that in rabbits this protein is an acute phase reactant. AOAH is a leukocyte enzyme and it functions in the clearance of LPS in a manner quite differently to those seen above. It detoxifies endotoxins by selectively removing the 2'and 3' secondary fatty acyl chains from the lipid A region of bacterial LPS to produce a tetra-acyl moiety with a lipid A domain structurally identical to lipid IVa (85). Thus, AOAH modulates host inflammatory responses to gram-negative infection by functioning as a lipase and deacylating LPS, converting the toxic lipid A moiety into a potent LPS antagonist, following phagocytosis (85).

It is evident from the above overview of LPS-binding proteins, that the host has indeed evolved an array of mechanisms in order to deal with bacterial infection. A lot of work has been done in the last decade to better understand

LPS action and mechanism in order to deal with bacterial infection. However, a lot more needs to be accomplished to provide a better treatment for sepsis.

Potential Treatments for Septic Shock

In order to study septicemia and to provide therapies for patients with sepsis, a thorough understanding of the LPS structure-function and mechanism of action within the host is essential. Only then can experimental approaches to block septic shock be provided. However, the complex structure of the macromolecule LPS and it's interactions with cells, specifc and nonspecific, by no means makes this task easier.

Within the last couple of decades, several approaches have been taken to study the mechanism of LPS action. Scientists have gone about this essentially by either targeting LPS directly or by interrupting the cytokine-mediated immune response to septicemia.

In the first strategy, LPS in the blood can be neutralized to prevent it from activating leukocytes that start the inflammatory response. In the 1970s, work on LPS, done by the late Abraham Boude (49) led to the first attempts by Elizabeth Ziegler and her colleagues, almost a decade later, to halt septic shock. In a study done by this group half the number of patients, with gram-negative septicemia, receiving human antisera to a mutant strain, *E.coli* J5 lived compared to those who received the control sera. However, one of the major problems with human antisera from many different donors is the high probability of the antiserum also

transmitting infectious agents. The other problem being that different human donors have varying immune responses to the test antigen, therefore, the approach cannot be rigidly standardized for effectiveness. Furthermore, the complex structure of LPS with the presence or absence of the variable oligosaccharide side chains does makes the production of antiserum not only an inconvenient, but, also sometimes an ineffective approach.

In the mid 1980s monoclonal antibodies were manufactured, which according to the manufacturers, would solve the problems of quantity, quality and contamination and provide a new means to block septic shock. However, other recent studies have shown that the mAbs against the lipid A region are not always effective, because based on the chemical composition of LPS, the lipid A region is not easily accessible as a potential target for therapy. In addition to the above difficulties, one such mAb produced by a bio-technology company (Centocor) has been shown to be toxic (49).

With the advent of the recent identification of several LPS responsive and resistant cell lines, identification of new LPS-binding protein(s) and a better understanding of the function(s) of previously identified LPS-binding proteins, more options are currently available that can potentially be used for therapeutic purposes (2, 12, 24, 25, 42, 53). Recombinant NH_2-terminal fragment of BPI, monoclonal antibodies against cytokines such as TNF-α, IL-1, IL-6 and IL-8, soluble TNF-α, IL-1 receptors and new monoclonal antibodies to LPS (10, 30, 35,

110), have been shown to be useful and are being used currently in different phases of clinical trials (7, 16, 49).

Because, of the great complexity of the interaction of LPS with the immune system and the resulting cascade of events, it is unclear whether inhibition of a single mediator will prevent the pathogenesis of sepsis. Thus, a lot more work needs to be done before pathogenesis due to bacterial infections can be controlled. However, with more research being done on the action of LPS, newer approaches can be undertaken to block sepsis. Thus monoclonal antibodies against LBP and CD14 could possibly be used as potential therapeutic agents in the treatment of gram-negative bacterial septicemia. Studies carried out in domestic animals to date have provided invaluable knowledge in the study of septic shock and the role of LBP and CD14.

Like any other species, cattle are very sensitive to bacteria and pathogenesis caused by gram-negative bacteria accounts for a substantial loss of dairy calves and represents a major loss to the cattle industry (9, 13). Usually very young calves are most likely to be affected by *E. coli* induced septicemia, and this proves to be rapidly fatal. Slightly older confined dairy calves, on the other hand, are more affected by *Salmonella sp.* than *E. coli*. And, systemic spread of bacteria to the liver, lung, and other organs often accompanies primary infection of the gastrointestinal tract (9). Studies have shown that administration of calves with antisera to *E. coli* and bacterial LPS does not always prove to be effective, even though the survival of neonatal calves is dependent upon passive transfer of

sufficient quantity of immunoglobulins (9). This suggests that research at immunoregulation should focus on factors other than targetting at LPS itself.

Macrophages have been shown to play an important role in release of cytokines and host defense and several studies have shown that the interplay between these cytokines and macrophages is of great significance (11, 94). Thus a better understanding of the action of LPS on macrophages is crucial. To date, published literature on LPS-binding proteins does not include research done using the bovine system even though bovine sera and cells are widely used in research. A few years ago, *in vitro* studies done by Adams et. al. (1) have shown that bacterial LPS elicits TNF-α from bovine leukocytes. However, the protein(s) involved, if any, in the induction of bovine TNF-α have not been previously identified. In other words, the mechanism of LPS action in bovine cells remains to be clearly defined. Since, the presence of any protein(s) in bovine sera or cells can influence experimental results of users of the bovine serum and cells, it is imperative that LPS-binding protein(s) be identified from bovine sera and researchers become aware of their existence.

CHAPTER 2

HYPOTHESES AND OBJECTIVES

A. LBP exists in bovine serum and bovine mononuclear phagocytes respond to LPS-LBP complexes.

B. LPS-LBP complexes enhance the sensitivity of mononuclear phagocytes to LPS and these cells respond to LPS-LBP complexes in a proinflammatory manner.

C. CD14 on the surface of bovine mononuclear phagocytes acts a receptor for LPS-LBP complexes, and that LPS-LBP complexes bound to CD14 enhance the sensitivity of mononuclear pagocytes to LPS.

GOALS AND OBJECTIVES

The goals and objectives of this research project are:

1. To identify, isolate and characterize bovine LBP from bovine serum. Bovine LBP has not been previously isolated, and, the specific objectives include fractionation of bovine serum, using ion exchange chromatography, and characterization of proteins that specifically bind radio-labeled LPS.

2. To generate specific polyclonal antisera to bovine LBP and to use LBP-enriched fractions to investigate effects of LPS-LBP complex on modulation of macrophage sensitivity to LPS. Specific objectives include conducting comparative bioassays of LPS-mediated macrophage activation with/without LBP containing fraction.

3. To study the mechanism of action of LPS in bovine alveolar macrophages. Specifically, to show that LPS-LBP complex functions through the membrane bound CD14 receptor on the alveolar macrophages. This will be demonstrated by performing cell assays in which macrophages will be stimulated with LPS-LBP complex with/without monoclonal antibodies to CD14.

CHAPTER 3

EXPERIMENTAL DESIGN AND SIGNIFICANCE

LPS-binding molecular species(s) will be detected by using isopycnic ultracentrifugation technique. This method has previously been used for the detection of LBP in other systems (96, 97). Briefly, labeled LPS will be incubated with normal and bovine serum obtained post-LPS treatment and the LPS-binding molecular specie(s) will be identified by running CsCl isopycnic density gradients. LBP is expected to have a density of 1.30 gm/cm ^{3}. Upon the detection of LBP binding activity at the above density, studies will be done to show specificity of LPS binding. This will be demonstrated by using varying concentrations of unlabeled LPS in the presence of a fixed concentration of labeled LPS. Based upon the results obtained from the above experiments either normal or acute phase bovine serum will be used to purify LBP. This will be done by using previouly published methodology (98). Briefly, using ion exchange chromatography bovine serum will be fractionated. SDS-PAGE will be performed to show the purity of the obtained protein fractions. LPS-binding activity will be demostrated by performing photoaffinity cross-linking studies using ^{125}I-ASD-LPS. In order to show the specificity of this binding, ^{125}I-ASD-LPS will be covalently linked to LBP containing fractions in the presence and absence of unlabeled LPS. Radiolabeled protein fractions will be run on SDS-PAGE gels. Radiolabeled BSA will be used as a molecular weight marker along with prestained molecular weight

standards and autoradiography will be performed. Results from these studies should show the presence of a specifically labeled protein of an apparent molecular weight of 60 kDa.

At this stage, the fraction containing protein that binds LPS specifically will be identified and that fraction will once again be run on a SDS-polyacrylamide gel and proteins will be transferred onto a nitrocellulose sheet (103). Proteins will be visualized using commassie brilliant blue and the band of interest will be cut out and used for NH_2-terminal sequencing.

Upon observing the desired homology between the bovine 60 kDa LPS-binding protein and the other previously characterized family of LBPs (102), the fraction containing the protein of interest will be run on a SDS-acrylamide gel, proteins will be visualized using commassie brilliant blue and the band that binds LPS specifically will be identified and cut out. This band will be used to generate polyclonal antibodies in rabbits.

The next step will involve using HPLC to further purify LBP. Once again purity of the collected fractions will be determined by performing SDS-PAGE. Next cross-linking studies will be performed on fractions collected to show whether the collected fractions have retained their LPS-binding activity.

The biological activity of the fractionated and HPLC purified 60 kDa LPS-binding proteins will be shown by performing tissue factor assays. These studies will be done in the presence and absence of physiologically relevant concentration of LPS. Other assays will include anti-human CD14 monoclonal antibodies,

polymyxin B and heat treated fractions containing the 60 kDa LPS-binding protein. These studies should demonstrate that a protein fraction isolated from bovine serum contains a protein that functions in a manner analogous to the LBPs previously isolated from rabbit, human and bovine serum (102, 105), That is through the membrane bound CD14 receptor on bovine alveolar macrophages. These studies will also show whether this protein is heat labile and if it binds to the lipid A region of LPS.

SIGNIFICANCE

The goal of the proposed research is to purify and characterize bovine LBP, a protein that has been proposed to play an immunomodulatory role. Thus the purification and biochemical characterization of LBP will enable future studies of pathogenesis on the cellular and molecular level. The signalling pathways in LPS stimulated macrophages will be of particular interest. Furthermore, since gram-negative sepsis/septic shock continues to plague cattle and other vertebrates, this research should lead in the direction of future rational treatments for disease.

Contributions to Further Research:

(1) Polyclonal antibodies to bovine LBP could be used to prepare an affinity column which would enable purification of bovine LBP with more efficiency.

(2) The antibodies developed against bovine LBP could also be used to screen the bovine liver lambda gt11 cDNA library to isolate a full length clone. This would impart the knowledge of the entire DNA and protein sequence of bovine LBP.

(3) Using site-specific mutagenesis next one could determine the region(s) of importance for LPS binding, reactions associated with LPS binding to LBP and those for the binding of LPS-LBP complex to CD14.

(4) The above information could be extremely valuable in preparation of drugs to manipulate the potentially deletrious and lethal effects of LPS on the host.

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APPENDIX

FIG. 1 (A). Schematic molecular representation of the *E. coli* envelope. Ovals and rectangles depict sugar residues. Circles represent the polar headgroups of phospholipids. MDO are membrane-derived oligosaccharides, and KDO is 3-deoxy-D-manno-octulosonic acid. KDO and heptose make up the inner core of LPS.

Source: Raetz, C. R. H. (1990) Biochemistry of Endotoxins. *Annu. Rev. Biochem.* 159:129-170.

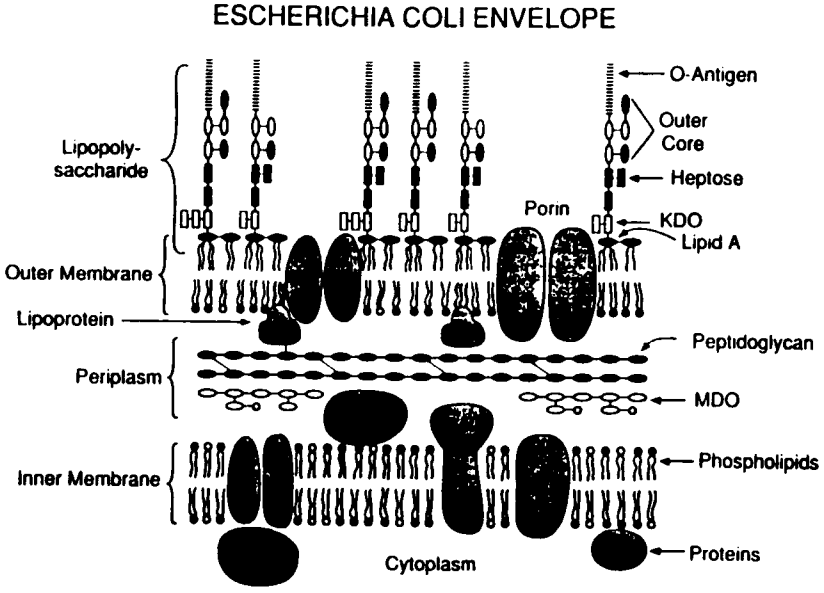


FIG. 1 (B). Schematic of the chemical structure of endotoxin.

Source: Rietschel, E. T. and H. Brade (1992) Bacterial Endotoxins. *Sci. Amer.* *267*:54-61.

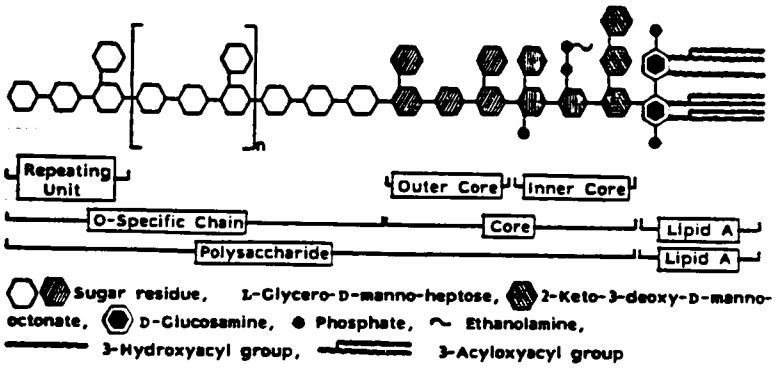


FIG. 2. Role of multiple receptors in the response to LPS.

Source: Wright, S. D. (1991) Multiple receptors for endotoxin. *Curr. Opin. Immunol. 3:83-90.*

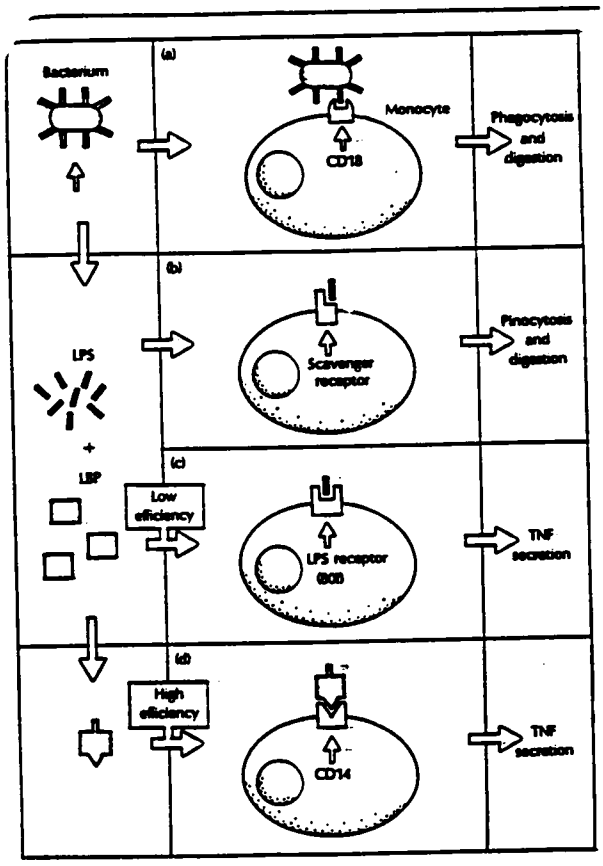
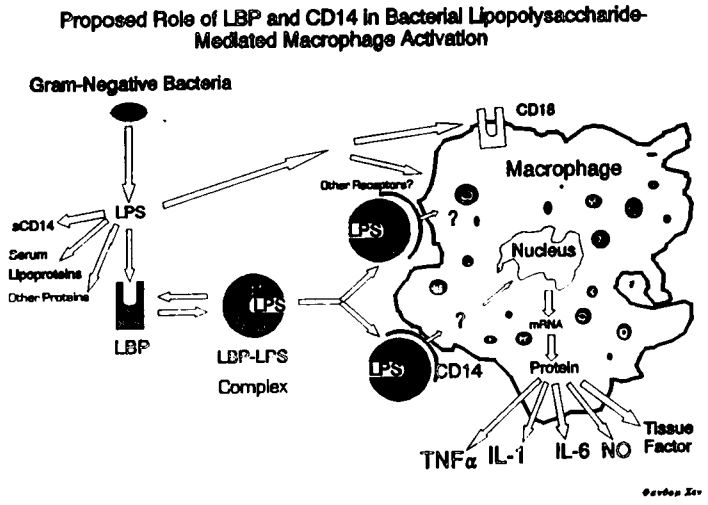


FIG. 3. Proposed role of LBP and CD14 in the activation of macrophages by LPS.

Source: Raetz, C. H., R. J. Ulevitch, S. D. Wright, C. H. Sibley, A. Ding, and C. F. Nathan (1991) Gram-negative endotoxin: an extraordinary lipid with profound effects on eukaryotic signal transduction. *FASEB J.* 5:2652-2660.



PART 2

LIPOPOLYSACCHARIDE-BINDING FACTORS ARE PRESENT IN BOVINE SERUM

ABSTRACT

Response of vertebrates to bacterial lipopolysaccharide may be regulated, in part, by serum factors that influence bioavailability and cellular binding affinity of lipopolysaccharide. Using [^{3}H]- or [^{14}C]-lipopolysaccharide, or a novel fluorescence-based assay for detection in CsCl isopycnic density gradients, our studies indicate existance of factors in adult and fetal bovine serum that bind LPS. Within serum-containing gradients, labeled LPS appeared in two peaks: least-dense fractions ($\leq$1.30 g/cm^{3}), and at 1.35 g/cm^{3}. This profile was different than those of gradients without serum, where LPS appeared at 1.38 g/cm^{3}. Binding of LPS to serum component(s) at 1.35 g/cm^{3} was rapid (< 1 minute), saturable, and specific. A partial shift (50%) of LPS from a density of 1.35 g/cm^{3} to other serum components at $\leq$1.30 g/cm^{3} occurred over 1 hour. Flow cytometric analysis indicated that bovine serum factors influence binding of LPS to blood monocytes, because monocyte-FITC-LPS association increased in the presence of bovine serum.

CHAPTER 1

INTRODUCTION

Deleterious and lethal consequences of gram-negative bacterial infection with systemic release of bacterial lipopolysaccharide (LPS; endotoxin) have been well documented in many species (5, 6). LPS plays an important role in the pathogenesis of endotoxic disease induced by common gram-negative bacterial pathogens, such as *Escherichia coli* and *Salmonella* sp. A key interaction in this pathogenesis is the association of LPS with monocytes and macrophages. LPS induces enhanced synthesis and secretion of many mononuclear phagocyte-derived proinflammatory and immunologically active cytokines, such as IL-1, IL-6, and TNF-α, which are central to the polysystemic manifestations of endotoxic disease (1, 3, 7). Factors that affect initial binding of LPS to monocytes and macrophages may thus play a pivotal role in determining the outcome of LPS exposure.

Lipopolysaccharide binding protein is a recently described acute phase protein synthesized in response to acute inflammatory stimuli (9, 12). LBP present in serum forms high-affinity complexes with LPS, and LPS-LBP complexes bind to mono-nuclear phagocytes through a specific receptor, CD14 (14). Exquisite host sensitivity to extremely low levels of circulating LPS may result from this interaction, and this may facilitate recognition of trace quantities of LPS early in the course of infection. Experimental evidence exists that some serum factors may bind and thus influence the bioactivity of LPS (2, 10, 13), and

therefore probably influence the pathogenesis of LPS-mediated disease. Many researchers utilize bovine sera, cells, or tissues for in vitro studies, but published work in this field does not address LPS-binding activity in bovine sera. Because this serum activity may influence certain experimental results, our objective was to demonstrate and characterize LPS-binding activity in bovine sera.

CHAPTER 2

MATERIALS AND METHODS

Materials

Fluorescinated (FITC) LPS from *E.coli* 0111:B4 was purchased from Sigma Chemical CO. (St. Louis, MO., USA) and [^{14}C]LPS from *S. typhimurium* Rc was from List Biological Laboratories (Campbell, CA., USA) [^{3}]LPS Salmonella minnesota Re595 was a gift from Dr. Peter Tobias. All other reagents were purchased from Sigma Chemical CO. (St. Louis, MO., USA). A Beckman vT65i rotor was used for centrifugation. A Shimadzu RF5000U spectrofluorphotometer (Japan) and a Becton-Dickinson FACScan was used for analysis of fluoresence.

Serum Samples

Sera was obtained from healthy adult cows, both before and after (24, 48, 72 h) intravenous infusion of 1 μg/Kg LPS from *E. coli* 0111:B4. Whole blood collected by jugular venipuncture was allowed to clot for 1-2 h at 37℃, centrifuged (20 min, 1200 g, 22℃), the clots compressed, and similarly re-centrifuged. Supernatant serum was then collected and centrifuged (30 min, 2200 g, 22℃) to remove particulates, then frozen without preservative (-70℃) until use. Blood was also obtained by otic arterial phlebotomy or cardiac puncture from both normal New Zealand White rabbits and from rabbits in which an acute inflammatory reaction had been induced by subcutaneous injection of silver nitrate solution (3% wt/vol)

as described (12). The serum was harvested and similarly stored frozen without preservative. Pooled fetal bovine serum was kept frozen until use.

CsCl Isopycnic Density Gradients

FITC-LPS from *E. coli* 0111:B4 (5.7 μg FITC/mg), [^{3}H]LPS from *Salmonella minnesota* Re595, or [^{14}C]LPS from *S. typhimurium* Rc was resuspended in 10 mM EDTA (pH 7.4) and added (20 μg/ml) to 0.4 or 0.5 ml of serum (in the presence of 10 mM EDTA) and allowed to incubate for 10 minutes at 37℃ (12). This step was modified for kinetic studies by varying the incubation period from 1 min to 6 hr. Modification of this step for total and nonspecific binding studies included incremental addition (20, 100, 400 μg/ml) of unlabeled LPS. Saturation studies included addition of labeled LPS in incremental amounts (10, 20, 40, 100, 240, 350, 500 μg/ml) Quantity of serum was also varied from 0.125-0.5 ml in certain experiments. The reaction was stopped by addition of cold CsCl (2.81 M CsCl in most cases in 0.15 M NaCl, 4℃. 2.4 M CsCl also used where indicated), and then centrifuged to equilibrium for 16 hours at 45,000 RPM and 4℃, using a vertical tube rotor (VTi 65, Beckman). After centrifugation, the gradients were fractionated into 12-14 samples and analyzed for the presence of LPS utilizing either liquid scintillation counting or fluorescence spectroscopy. The presence of FITC-LPS was detected using a Shimadzu RF5000U spectrofluorophotometer, with excitation at 480 nm and the emission analysis range from 470 to 700 nm. Slit widths for excitation and emission were typically 3 nm. Resultant spectral

peaks were integrated using proprietary software (Shimadzu) and reported as spectrum integration units. Density profiles of the gradients were determined utilizing refractive indices and are reported as g/cm ^{3}. Density-gradient profiles of LPS in the absence of serum were obtained using LPS (20 μg/ml) in 0.15 M NaCl, pH 7.4.

Flow Cytometric Analysis of Peripheral Blood Monocytes

Bovine blood was collected from clinically normal adult cattle by jugular venipuncture. Complete blood counts including differential leukocyte counts were performed on each sample to ensure conformance with normal values. Anticoagulated blood (acid citrate dextrose, 1:10) was centrifuged (700 g, 15 min) and the buffy coats collected. After flash hypotonic lysis of erythrocytes, the leukocytes were washed three times by suspending them in Hank's Buffered Salt Solution and centrifugation (200 g, 10 min). Final preparations were composed of 8-15% monocytes as determined by cytospin preparations, with the remainder consisting of primarily lymphocytes and small numbers of neutrophils and eosinophils. Mixed leukocyte preparations (1 X 10^7/ml, 2.0 ml total) were labeled with Dil-Ac-LDL1 (10 μg/ml; Biomedical Technologies Inc.) for 90 min. at 37°C with slow, end-over-end mixing. Other control cells were similarly incubated without the label. After incubation the cells were chilled, centrifuged (200 g, 10 min), and resuspended in FITC-LPS (*E.coli* 0111:B4, 10, 100, or 1000 ng/ml), with or without bovine serum. Cells were then similarly incubated for 90 min.,

centrifuged, resuspended in HBSS and fixed with 1.0% paraformaldehyde. Quantitative fluorescence analysis was accomplished utilizing a Becton-Dickinson FACScan, with analysis gates set to collect and quantitate the FITC fluorescence (LPS) associated with monocytes (large forward scatter and positive for Dil-Ac-LDL red label.) Any cellular autofluorescence was subtracted from relative fluorescence values, and 10,000 cells were counted per treatment.

CHAPTER 3
RESULTS

CsCl Isopycnic Density Gradients

LPS-binding activity at 1.35 g/cm ^{3} in CsCl gradients was detected in sera from adult bovines and from fetal bovine serum, and shared some similar characteristics to patterns of LPS-binding activity observed by others using rabbit serum that contained lipopolysaccharide binding protein (10, 11, 12). In this work, lipopolysaccharide binding protein in rabbit acute phase serum formed complexes with [^{3}H]LPS at a density of 1.30 g/cm ^{3} in CsCl isopycnic density gradients, and serum lipoproteins also bound LPS producing a peak at $\leq$1.20 g/cm ^{3}. In Figure 1 are similar results from our laboratory using acute-phase rabbit serum, with a major peak at 1.32 g/cm ^{3}, and a second similar peak in the least-dense fractions. Bovine serum also yielded qualitatively similar results, but the major non-lipoprotein LPS complex appears at a density of 1.35 g/cm ^{3}. [^{3}H]LPS in the absence of serum appeared in the most dense fractions (1.38 g/cm ^{3}). Results obtained utilizing [^{14}C]LPS derived from *S. typhimurium* were similar.

Fluorescence spectroscopy proved useful for analysis of FITC-LPS binding to bovine serum components. Quantitative spectral analysis demonstrated a linear relationship between the concentration of FITC-LPS and resultant fluorescence intensity (Figure 2). When using a spectrofluorophotometer slit width of 3 nm,

FITC-LPS (5.7 μg FITC/mg LPS) could be detected at concentrations as low as 1 μg/ml. Sensitivity was enhanced beyond this lower limit by increasing the slit width. Qualitatively similar results were obtained using either fluorescence or radioisotope-based methodology, and the LPS-serum complex at 1.35 g/cm ^{3} was detected in bovine serum derived from both untreated and LPS-treated adult cows, and in commercial fetal bovine serum. Sera from different animals exhibited consistent LPS-binding activity at 1.35 g/cm ^{3}, but the spectrum integration values varied by as much as 2.4-fold (data not shown). FITC-LPS is readily available from commercial sources.

Varying the amount of bovine serum allowed to react with FITC-LPS resulted in proportionally more or less complex formation at 1.35 g/cm ^{3} (Figure 3), consistent with the presence of serum factors that bind LPS. Total and nonspecific-binding assays indicated that the bovine serum factors responsible for complex formation at 1.35 g/cm ^{3} bind specifically to LPS (Figure 4). Saturation curves indicated that the serum factors at 1.35 g/cm ^{3} are saturable, with a Km of 150 μg FITC-LPS/ml (Figure 5). In contrast, FITC-LPS association with serum factors in the least-dense fractions ($\leq$1.30 g/cm $^{3)}$ was not saturable at concentrations up to 500 μg FITC-LPS/ml. Kinetic studies indicated that FITC-LPS serum complex formation at 1.35 g/cm^3 occurs in as little as one minute (Figure 6). There was a progressive shift of FITC-LPS from complex 1.35 to the least-dense fraction ($\leq$ 1.30 g/cm^3) over 1 hour, but further shift did not occur by 6 hours. This

time-dependent LPS shift also occurs in rabbit serum, from lipopolysaccharide binding protein to serum lipoproteins (12).

Flow Cytometric Analysis of FITC-LPS Binding to Monocytes

Flow cytometric studies indicated that serum components enhance binding of LPS to peripheral blood monocytes (Figure 7). Association of FITC-LPS with monocytes was enhanced in the presence of 10% bovine serum by a minimum of 5-fold over a range of LPS concentrations, compared to LPS in the presence of buffer alone. Studies have not included specific bovine serum components, and the identity of serum components that promote LPS-monocyte association have not been demonstrated. However, considering the known proinflammatory effect of LPS on mononuclear phagocytes, such as peripheral blood monocytes, the ability of serum components to induce and markedly enhance LPS binding is worthy of note.

CHAPTER 4

DISCUSSION

The interplay between pathogenic gram-negative bacteria, bacterial LPS, mononuclear phagocyte activation with synthesis of proinflammatory cytokines, and the pathogenesis of endotoxemia and endotoxin-related disease is of great biomedical interest. Bacterial LPS is a remarkable lipid, inducing generation of cytokines such as IL-1 and TNF α from LPS-activated mononuclear phagocytes, and these cellular intermediaries are key components of the biologic response to LPS (4, 8). Resultant sequelae of LPS-induced monocyte/macrophage activation and release of cytokines may include enhanced host defense, but adverse pathophysiologic effects and death also occur. Factors that affect or modulate the interaction of LPS with mononuclear phagocytes are, therefore, important in the pathogenesis of LPS-mediated disease. The use of fluorescence for detection of LPS-bovine serum interactions in CsCl gradients proved reproducible and convenient. FITC-LPS conjugates derived from a number of gram-negative bacterial sources are available, and provide readily available material for further studies in this area.

In our studies we have demonstrated the presence of bovine serum factors that bind bacterial LPS, and that bovine serum remarkably enhances association of LPS with monocytes. Our studies have indicated some similarities between the bovine serum factor(s) at 1.35 g/cm ^{3} and reported characteristics of rabbit

lipopolysaccharide binding protein (12), but the identity and characteristics of the bovine serum factor(s) remain to be elucidated. These findings have notable implications for researchers utilizing bacterial LPS, and sera or other biological fluids derived from bovine sources. The bioavailability and bioactivity of LPS may vary in accordance with the presence of LPS-binding factors present in bovine serum or fluids, and potentially affect the outcome of experimental assays.

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APPENDIX

FIG. 1. [^{3}H]LPS with either rabbit acute phase serum or bovine post-treatment serum (LPS injected) in CsCl density gradients (2.4 M). The peak LPS complex formation in rabbit fraction 6 is at 1.32 g/cm ^{3}, and the peak LPS complex formation in bovine serum in fraction 3 is at 1.35 g/cm ^{3}.

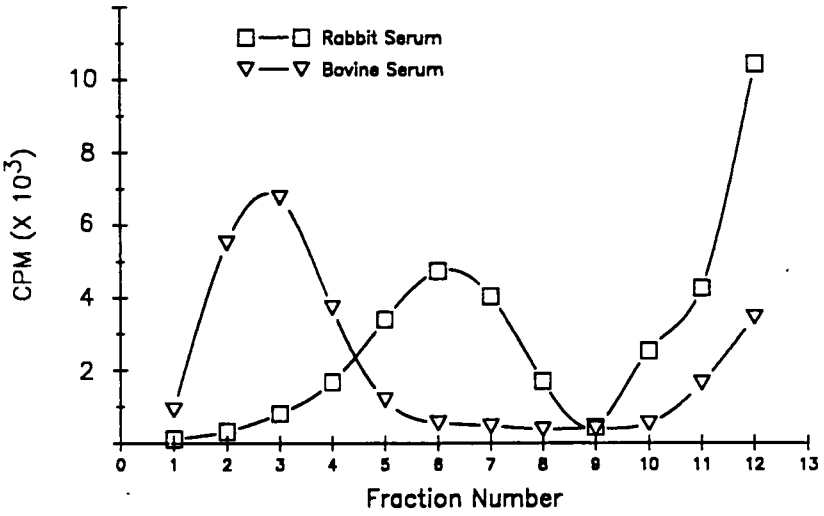


FIG. 2. Fluorescence intensity of FITC-LPS is linear over a range of LPS concentrations.

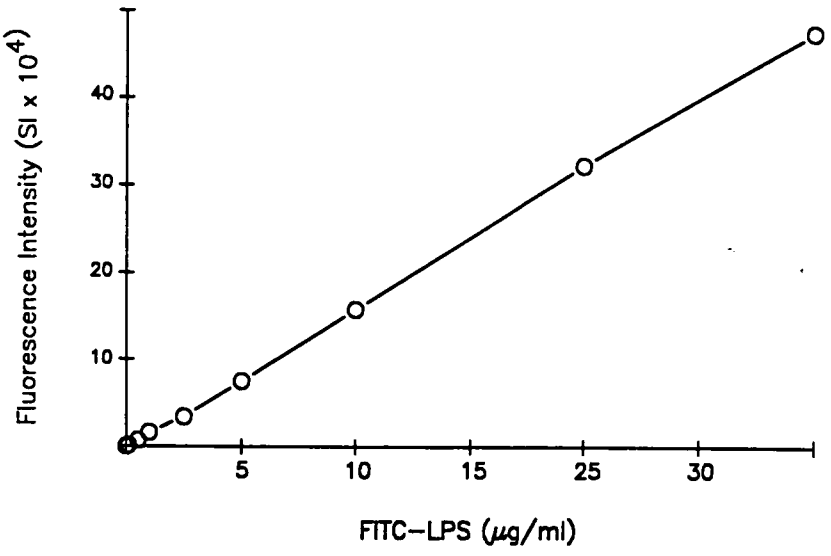


FIG. 3. The quantity of FITC-LPS bound to serum components at 1.35 g/cm ^{3} in CsCl gradients is proportional to the amount of bovine serum present. FITC-LPS concentration was held constant at 20 μg/ml. Dotted line indicates CsCl gradient.

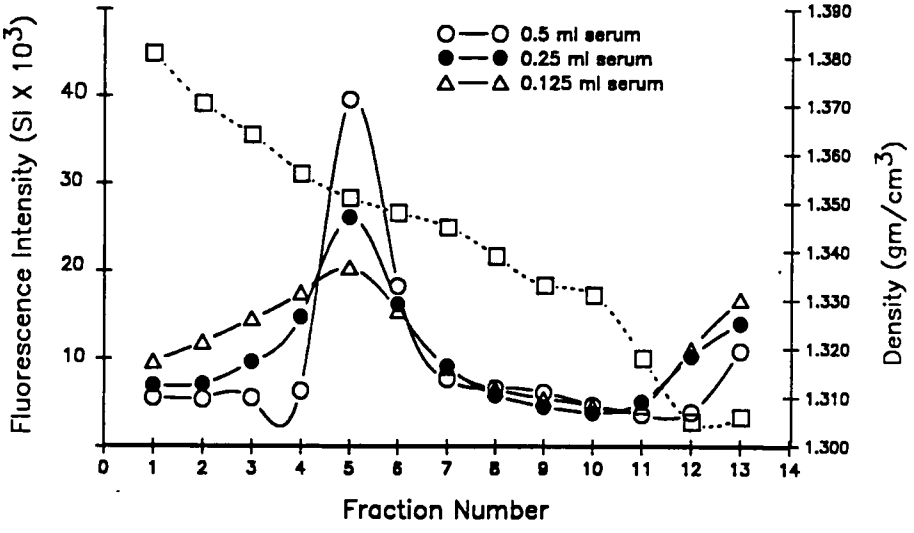


FIG. 4. Competitive-binding assay. FITC-LPS complex formation in fraction 5 of CsCl gradients (1.35 g/cm ^{3}) was reduced in the presence of excess unlabeled LPS.

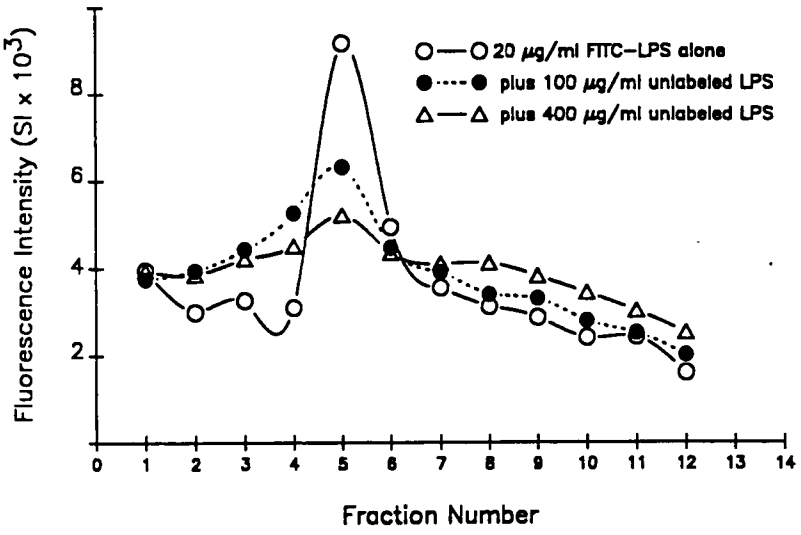


FIG. 5. LPS-complex formation with bovine serum components at 1.35 g/cm ^{3} was saturable; Km was 150 μg/ml FITC-LPS. LPS-complex formation at a gradient density $\leq$1.30 was not saturable at LPS concentrations up to 500 μg/ml

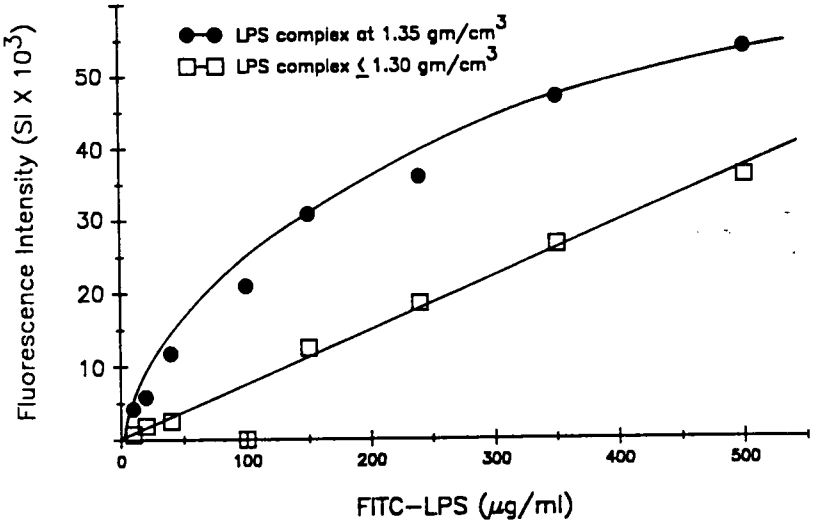


FIG. 6. Kinetics of LPS binding to bovine serum components. Binding of FITC-LPS to serum component(s) appearing at a density of 1.35 g/cm ^{3} in CsCl gradients occurred in as little as 1 min, and there was a progressive shift of LPS to less dense serum components during 1 hour of incubation.

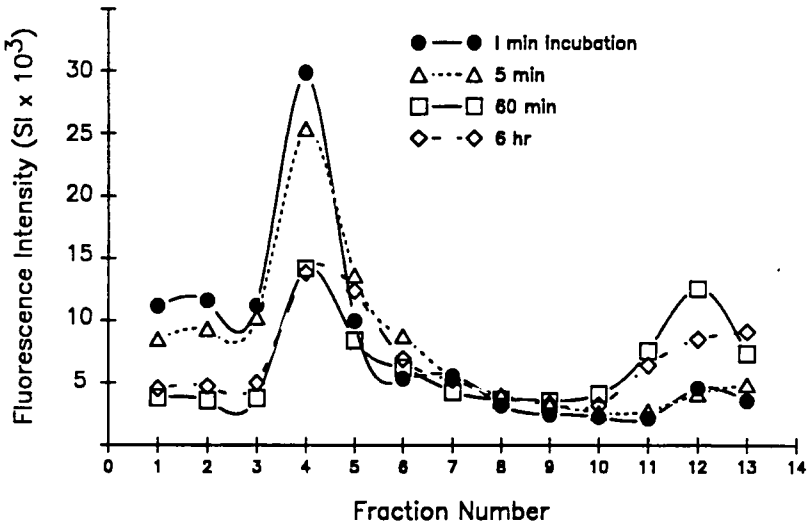
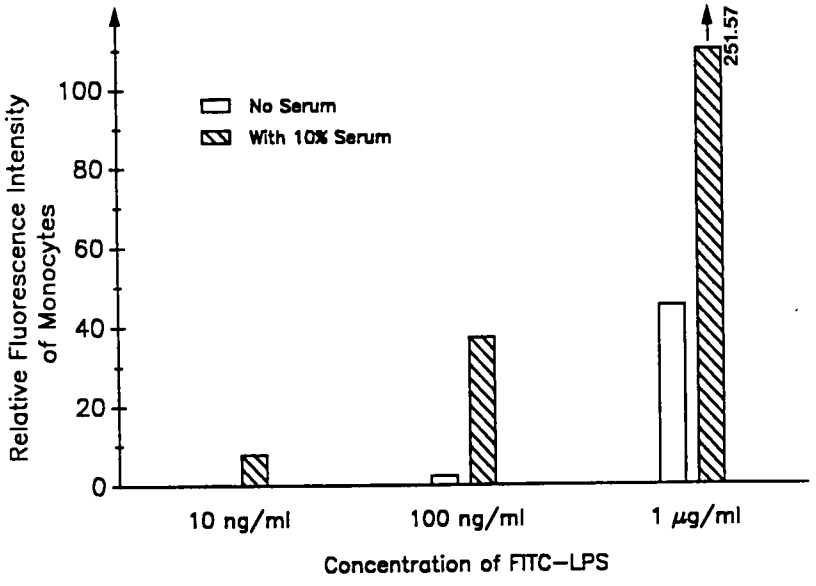


FIG. 7. Binding of FITC-LPS to peripheral-blood monocytes. Fluorescence flow cytometric analysis indicated that the presence of 10% bovine serum markedly enhanced association of FITC-LPS with monocytes. Sera from fetal bovines (10%) yielded similar results. Ordinate scale is relative green (FITC) fluorescence. Fluorescence intensity without serum was negligible at 10 ng/ml FITC-LPS. Typical results from one of four assays is shown.



PART 3

PURIFICATION AND CHARACTERIZATION OF BOVINE LIPOPOLYSACCHARIDE-BINDING PROTEIN

ABSTRACT

Endogenous regulatory mechanisms exist in mammals which enable a rapid response to lipopolysaccharide (LPS, endotoxin) emanating from gram-negative bacterial infections. Serum proteins and cell-surface receptors exist that bind LPS, and this interaction may either aid in nonpathogenic removal of LPS from the body, or cause significant potentiation of the effects of LPS on the immune system. We have used a photoreactive, thiol-cleavable, radiolabeled derivative of *E.coli* 0111:B4 LPS, LPS-(*p*-azidosalicylamido)-1,3'-dithiopropionamide (LPS-ASD) to identify the presence of LPS-binding proteins in bovine serum. Ion-exchange chromatography was used to fractionate bovine serum, and eluted protein was subsequently photoaffinity labeled using ^{125}I-ASD-LPS. LBP(s) were identified by autoradiography of sodium dodecyl sulfate-polyacrylamide gels. Three LBPs with apparent molecular masses of 65, 60 and 50 kDa were present within one of the chromatography pools. Binding of ^{125}I-ASD-LPS to the 60 kDa bovine LBP was inhibited in the presence of homologous underivatized LPS, indicating specificity of LPS binding. The *N*-terminal amino acid sequence of the 60 kDa protein showed 77% homology with human LBP, and 68% with rabbit LBP. Our results indicate that an LPS-binding protein exists in samples of pooled bovine serum, and that this protein has features in common with human and rabbit LBP.

CHAPTER 1

INTRODUCTION

Lipopolysaccharide (LPS, endotoxin) is an integral component of the cell wall of gram-negative bacteria and has been demonstrated to play an important role in the pathogenesis of septicemia induced by gram-negative bacteria (8,12,14,15). Systemic release of bacterial LPS may induce deleterious or lethal consequences in many species (6, 7, 12), and recent research has demonstrated several important regulatory molecules that augment or diminish the effect of LPS on the host. These endogenous regulatory mechanisms within the bloodstream interact with LPS and include several components of serum such as albumin (11), high and low density lipoproteins (11, 20), LPS-binding protein (LBP) (9, 11, 16-18, 21-23, 27, 28), soluble CD14 (2, 19) and septin (29).

It has been demonstrated that while LBP is present in normal serum, concentrations increase in response to acute inflammatory stimuli. LBP in serum binds LPS with high affinity (21), and the resultant LBP-LPS complex binds to CD14 receptors (27, 28) on the surface of monocytes and macrophages. This interaction enables these leukocytes to respond to profoundly lower concentrations of LPS compared to LPS alone (4, 18, 26), and the LPS threshold for generation of proinflammatory cytokines such as TNF-α, IL-1, and IL-6 is much lower (4, 5, 12). 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 (14, 15). Factors that affect or modulate the interplay of LPS and its target cells are therefore important in the pathogenesis of LPS-mediated diseases, and the presence of these factors is also important in the design and interpretation of in vitro experiments.

Bovine animals, like many other species, are sensitive to the presence of bacterial LPS and suffer from a variety of gram-negative bacterial infections, and we have previously demonstrated the presence of uncharacterized LPS-binding factors in bovine serum (9). Aside from interest in bovine LBPs because of gram-negative bacterial disease in this species, bovine sera or cells are widely utilized for in vitro studies by many researchers. In our current work, we have extended our observations with findings that indicate the presence of multiple LPS-binding proteins in bovine serum, and we have identified a 60 kDa LBP that binds with specificity to LPS from *E. coli* 0111:B4. The 60 kDa LBP has significant N-terminal sequence homology to human and rabbit LBP. In addition, we have used a previously published method of HPLC (21) with slight modifications to isolate bovine LBP from serum protein mixtures.

CHAPTER 2

MATERIALS AND METHODS

Materials

LPS from *E. coli* 0111:B4 was purchased from List Biological Laboratories (Campbell, CA, USA), and sulfosuccinimidyl-2-(p-azidosalicylamido)1,3'-dithiopropionate (SASD) was from Pierce Chemicals (Rockford, IL, USA). Bio-Rex 70 resin, prestained molecular weight standards, polyvinylidene difluoride (PVDF) membrane (0.2 μm) and other materials for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) were obtained from Bio-Rad Laboratories (Richmond, CA, USA). Sephadex G-50 was purchased from LKB Pharmacia (Piscataway, NJ, USA). The ultrafiltration cell and YM10 membranes were obtained from Amicon Corp., (Danvers, MA, USA). Bovine serum albumin (BSA) was purchased from Sigma Chemical Co. (St. Louis, MO, USA), and Na ^{125}I from ICN (Lisle, IL, USA). The Mono-Q HR 5/5 column was purchased from Pharmacia Fine Chemicals, Piscataway, NJ.

Serum Samples

Sera were obtained by jugular venipuncture from healthy adult cows, pooled, and used for these studies. Animals serving as blood donors received the highest level of care, and all protocol were reviewed and approved by the University Animal Care and Concerns Committee. Whole blood was allowed to clot for 1-2 h at 37°C,

centrifuged (20 min, 1200 g, 22°C), the clots compressed and similarly re-centrifuged (30 min, 2200 g, 4°C). Collected serum was then similarly recentrifuged to remove particulates, and finally frozen without preservative (-70°C) until use.

Purification of LPS-binding protein

Bovine serum was first fractionated using Bio-Rex 70 column as previously published (21). Protein eluted from the column was monitored at 280 nm, samples were pooled into five fractions, and were dialyzed overnight against 5 mM Hepes, pH 7.3 (4°C). The fractions were next concentrated approximately 10 fold using an Amicon (YM 10 membrane) ultrafiltration cell. The concentrated protein was then aliquotted into 1 ml fractions and lyophilized using a speed vacuum (Jouan, RC1010, -60°C); concentrated protein was stored at -70°C until further use. These experimental methods have been repeated six times with similar results in our laboratory, using various pooled lots of serum obtained from several animals.

The second chromatographic step utilized HPLC (Waters), was performed according to the procedure described by Tobias et al. (21), with some modifications. A Mono-Q HR 5/5 column was used as the adsorbent, and the flow rate throughout the HPLC step was 1 ml/min. Briefly, the Mono-Q column was equilibrated with 20 mM diethanolamine buffer, pH 8.3. Injection of the sample was followed immediately by a 15 ml gradient of 0-50 mM ammonium sulfate in 20 mM diethanolamine, pH 8.3. The gradient was then steepened, going in 20 ml from 50 to 333 mM ammonium sulfate in the same buffer. Finally the column was washed

with 10 ml of 333 mM ammonium sulfate in 20 mM diethanolamine, pH 8.3 for 10 min. Elution of protein was monitored by absorbance at 280 nm and two fractions of 0.5 ml each were collected per min. Fractions of interest were first concentrated using a speed vacuum, then resuspended in 20 mM diethanolamine buffer, pH 8.3, and under similar (with the exception of the second, 50 to 333 mM, linear gradient which was accomplished in 15 rather than 20 min.) conditions to those above were rechromatographed on the Mono-Q column. In some instances, protein obtained from pool 2 (eluted using 220 mM NaCl) of Bio-Rex 70 column chromatography was photoaffinity labeled using ^{125}I-ASD-LPS prior to chromatography on HPLC (see next method).

In some instances, Mono Q column was washed with a lesser (20 ml instead of 40 ml or more) amount of 20 mM diethanolamine prior injection of protein.

Photoaffinity labeling

E. coli 0111:B4 LPS was first sonicated, then coupled to SASD as described previously (25). The resulting LPS-ASD complex was radiolabeled with Na^{125}I using Chloramine T as described (24, 25). The product typically had a specific activity of 6 x 10^{9}cpm/mg LPS. ^{125}I-ASD-LPS (0.5 μCi) containing 0.2 μg of LPS was then incubated with protein (60 μgs) from Bio Rex 70 chromatography pools in borosilicate glass tubes for 10 min at 37°C. Control incubations included BSA (60 μgs), and the others included radiolabeled LPS alone. Cross-linking of the ligand to the protein was then accomplished by photolysis for 10 min at room temperature by using short-wave

UV irradiation (Hoefer Scientific Instruments, CA., UVC 1000 cross-linker, maximal emission at 254 nm). After cross-linking, some samples were concentrated using a speed vacuum. Two different types of photoaffinity labeling studies were performed. The first photoaffinity labeling was done in the presence of a fixed amount of ^{125}I-ASD-LPS (1.0 μCi, containing 0.5 μgs of LPS) and various amounts of chromatography protein (6, 12, 24 and 36, and 48 μgs). The second photoaffinity labeling was done in the absence/presence of various amounts (10, 2, 1, and 0.5 μgs) of nonradiolabeled *E.coli* 0111:B4 LPS as a competitor to demonstrate inhibition of specific binding.

SDS-PAGE

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using the buffer system of Laemmli (10) with either a 8 or 10% resolving gel and a 4% stacking gel. The protein bands were visualized by Coomassie blue R250, silver staining, or by autoradiography. The relative mobilities of proteins were determined using prestained low-range molecular mass protein standards and BSA. When autoradiography was performed, gels were dried on a Slab gel drier (Hoefer) and Kodak X-Omat XAR-5 film was used with the aid of a Dupont Cronex Lighting Plus Intensifier Screen (X-Ray of Georgia, Norcross, GA, USA). Gels were kept at -- 70°C until (5-24 hr) the desired intensity was obtained.

Protein determination and amino-terminal sequencing of protein

Protein concentration was measured using the method of Bradford (Bio-Rad protein assay kit, Bio-Rad, Richmond, CA). Amino-terminal sequencing was done by gas-liquid phase sequential Edman degradation using an Applied Biosystems 473A sequenator, with an on-line PTH-AA HPLC analyzer model 120A (San Jose, CA, USA), with a reverse-phase C18 column. Protein bands were transferred from an unstained 8% SDS-polyacrylamide gel to PVDF membranes for 75 min by a previously described method (13). The membrane was then stained with Coomassie Blue, the protein band of interest (60 kDa) was cut out of the membrane, and the amino-terminal amino acid sequence determined.

CHAPTER 3

RESULTS

Fractionation of bovine serum using Bio-Rex 70 column chromatography

Fractionation of bovine serum using Bio-Rex 70 resulted in five protein peaks (pools) corresponding to increasing concentrations of NaCl in eluant buffers (Fig. 1A). Protein composition of these pools was examined by performing SDS-PAGE, under reducing conditions. As seen in Fig. 1B, many protein bands are present within each pool. The composition of proteins appeared to vary from pool to pool, however, indicating that this was a useful step in the initial separation of serum proteins. These experiments have been repeated six times with similar results, using various lots of serum from six different bovine animals.

Photoaffinity labeling of LPS-binding proteins

In order to identify LPS-binding activity within these pools, an aliquot from each pool was incubated with ^{125}I-ASD-LPS, cross-linked by exposing the sample to UV light, and analyzed by subjecting the sample to electrophoresis and autoradiography. As seen in the autoradiograph (Fig. 2), pools 1 (lane 2), 2 (lane 3), 3 (lanes 4 and 5), and 5 (lane 9) show LPS-binding proteins. Of these, only pools 2 and 3 show a 60 kDa LPS-binding protein, and pool 2 (lane 3) appears to have the greatest LPS-binding activity as evidenced by a more intense band. The proteins with apparent molecular masses of 30 and 40 kDa in lanes 4, 5, and 9 are

unidentified LPS-binding proteins. Based upon this data and previous identification of a 60 kDa LPS-binding protein in rabbit (21) and human serum (17), pool two was used for our further experiments. This experiment was done twice with similar results being obtained each time. In order to characterize the bovine LPS-binding proteins in pool 2, photoaffinity labeling of ^{125}I-ASD-LPS to proteins was done under various conditions. The first experiment involved binding and cross-linking studies done using constant amount of labeled ligand (^{125}I-ASD-LPS) and various amounts of protein from pool 2. The autoradiograph in Fig. 3 (lanes 2-6) shows the presence of three LPS-binding proteins with apparent molecular masses of 50, 60 and 65 kDa. As the protein amount is progressively decreased, going from left (lane 2, greatest) to right (lane 6, least), only the 60 kDa protein continues to bind significant quantities of ^{125}I-ASD-LPS. The arrow in Fig. 3 indicates the 60 kDa protein of interest. The second experiment was done by incubating a fixed amount of protein (pool 2) with various amounts of radiolabeled ligand. Under these conditions, the 60 kDa appeared to be more intensely labeled in the presence of the lowest amount (0.5 μCi, containing 0.2 μgs LPS) of ^{125}I-ASD-LPS. When the amount of radiolabeled LPS was increased, additional bands of varying molecular mass appeared (data not shown). Taken collectively, these results indicate the preferential interaction of ^{125}I-ASD-LPS with the 60 kDa protein. The above experiments were done twice with reproducible results obtained each time.

Inhibition of ^{125}I-ASD-LPS binding to LPS-binding proteins, in pool 2, by unlabeled LPS

The specificity of ^{125}I-ASD-LPS binding to proteins from pool 2 was determined by performing competition binding assays. In these assays a fixed amount of protein and radiolabeled ligand were incubated in the absence (total binding) and presence (nonspecific binding) of various amounts of homologous, unlabeled LPS (*E. coli* 0111:B4). As seen in the autoradiograph in Fig. 4 (lane 3), binding of the radiolabeled LPS occurs to at three proteins with molecular masses of 60, 50, and one of low molecular mass. Of these, ^{125}I-ASD-LPS binds in the greatest quantity to the 60 and the 20(+) kDa proteins. However, when the same incubation was done in the presence of excess unlabeled LPS (10 μgs), the 60 kDa band disappeared (lane 4). Additional incubations were done in the presence of lower quantities of unlabeled LPS (2, 1 and 0.5 μgs of unlabeled LPS), and our results (Fig. 4, lanes 5-7) show that in the presence of decreasing amounts of unlabeled LPS the 60 kDa band regains its intensity. These findings indicate that the interaction of LPS with the 60 kDa LPS-binding protein is specific. The above experiments were done twice with unambiguous blocking of the 60 kDa protein by unlabeled LPS each time.

N-terminal amino acid sequencing

Figure 5A represents an 8% SDS-polyacrylamide gel. In lane 1, protein from pool 2 of Bio-Rex 70 chromatography 60 has been run, and a 60 kDa Coomassie blue-

stained protein band appears (indicated by an arrow). This 60 kDa protein is not seen in chromatography pool 5 (lane 2) corresponding to the 1 M NaCl elution, as was the case for rabbit LBP (21). BSA (control) was run in lane 3. The identity of the 60 kDa protein band was assessed by obtaining a 22-residue N-terminal amino acid sequence. As seen in Fig. 5B, alignment of the obtained bovine LBP N-terminus sequence with the previously published sequences of LPS binding proteins from other species indicates considerable sequence homology. Bovine LPS-binding protein shares 77% sequence homology in this region to human LBP (17), 68% homology with rabbit LBP (21), and 50% homology with human bactericidal/permeability-increasing protein (BPI) (22). These results show that the 60 kDa bovine LPS-binding exists in pool 2 obtained from initial fractionation of bovine serum utilizing a Bio-Rex 70 column. The amino-terminus of the 60 kDa protein was sequenced twice with an identical sequence obtained each time.

Purification of bovine LPS-binding proteins using HPLC

Since a bovine protein of approximately 60 kDa with homology to rabbit and human LPS-binding protein was identified in the pool 2, this pool was used for further purification of the LPS-binding protein. Figure 6 (A and B) shows the HPLC elution profile of the protein chromatographed twice on the same column under slightly different gradient conditions (part of the gradient being steeper during rechromatography). It is seen that in the first run (Fig. 6A), two peaks

elute very close to each other, with retention times of 34.82 and 35.27 min. Rechromatography (Fig. 6B) of one of these fractions (# 70), resulted in further purification of the protein (LBP). This is evidenced by analyzing the electrophoretic migration of proteins in the collected fractions (Fig. 6C). A silver-stained SDS-polyacrylamide gel of the protein fractions collected from the first (lanes 3 and 4) and the second run (lanes 1 and 2) on Mono-Q column is seen Fig. 6C. These experiments were repeated 6 times with similar results. The choice of fractions to be used for rechromatography was based upon our previous experiments in which we photoaffinity labeled pool 2 proteins with ^{125}I-ASD-LPS prior to purifying them using HPLC. The elution profile of radiolabeled proteins purified on Mono-Q column looked similar to those obtained with unlabeled proteins. Upon counting the eluted fractions for radioactivity (data not shown) and by SDS-PAGE/autoradiography (Fig. 7), our results indicated which fraction should be rerun on HPLC. Lanes 1 and 2 show fraction numbers 67 and 68 obtained from rechromatography on the Mono-Q column. Note that lane 2 shows only one intensely labeled protein of apparently 60 kDa as opposed to the 60 and 30 kDa bands in lane 1. Below the 60 kDa bands in both the lanes is seen excess radiolabeled LPS. These experiments were repeated 3 times. For comparison, BSA used as a control in these HPLC experiments eluted at 20.1 min.

A difference in the protein elution profile was observed (Fig. 8A) when Mono Q column was washed with 20 ml of 20 mM diethanolamine as opposed to more than 40 ml with the elution conditions remaining identical to run 1. In order

to visulize proteins obtained by this purification, SDS-PAGE was performed and proteins were stained using coommassie blue (Fig. 8B). As seen from this figure, only lanes 5-7, representing protein pools 4-6 showed the 60 kDa band of interest.

CHAPTER 4

DISCUSSION

Bacterial lipopolysaccharides act as potent initiators of fever, shock, coagulation disorders, and other sequelae in humans and other mammals (6, 7, 8, 12, 14). LPS possesses little intrinsic cytotoxicity, but induces extraordinary proinflammatory changes in the host's own immune system, which may result in significant disorders. The high morbidity and mortality associated with Gram-negative bacterial infections prompted recent studies aimed at elucidation of biological molecules that interact with LPS, either diminishing or enhancing its effects. Tobias et al., (21) have shown the presence of a LPS-binding glycoprotein that is synthesized as a 50 kDa protein in hepatocytes and is released in rabbit acute phase serum as a 60 kDa protein. Results from other studies (23, 26, 27) have shown that the high affinity (Kd=nM range) and specific interaction of LPS-LBP is the first step in creating the link between LPS and CD14 receptors on surfaces of monocytes and macrophages. The interaction of LPS-LBP complex with surface CD14 is important in the resultant cytokine cascade. LBP enhances the sensitivity of the host to LPS, and it serves as a modulatory mechanism in the bioavailability of LPS to the host. A protein similar to rabbit serum LBP has also been identified in human serum (17).

LBP has not been previously identified and characterized in bovine serum. Our previous results (9) have demonstrated the presence of LPS-binding factor(s)

in bovine serum, and we now extend our observations by identification of bovine serum LBP. Using ^{125}I-ASD-LPS, a photoaffinity probe, we have identified several LPS-binding proteins of variable molecular masses (65 kDa, 60 kDa and 50 kDa) in bovine serum. Of the above proteins the 60 kDa protein binds LPS with apparent specificity as evidenced by our results obtained from competition studies. The 60 kDa protein binds LPS with higher affinity as compared to the 65 kDa and 50 kDa. This is evidenced by the appearance of the labeled 60 kDa band in the presence of the lowest protein concentration on autoradiographs and the preferential disappearance of the same band in the presence of unlabeled LPS.

The LPS binding proteins in rabbit and human serum that have been previously described are also approximately 60 kDa. In order to compare and further characterize the 60 kDa bovine LBP, the N-terminus sequence of the band was obtained. As seen from our results, there is considerable homology (77% and 68%) of bovine LPS-binding protein to both human (17) and rabbit (21) LBP. This further substantiates the presence of an analogous LPS-binding protein in bovine serum. Furthermore, the N-terminus sequence also shows homology to an endogenous LPS antagonist, the bactericidal/ permeability increasing protein (BPI) (22). Published sequences of a family of LPS-binding proteins (22) show significant homology even though the mechanism of action of these proteins is not necessarily similar (3, 18).

Initial fractionation of bovine serum in our studies using a Bio-Rex 70 column proved to be a useful first step in purification of LPS-binding protein,

because BSA and other proteins eluted in peak 1 (Fig. 1A and B), which allowed easier interpretation of photoaffinity labeling studies. Interestingly, much of the 60 kDa bovine LPS-binding protein eluted in peak 2 (obtained using 220 mM NaCl) as opposed to rabbit LBP which eluted in peak 5 (using 1 M NaCl) (21). Rechromatography with anion exchange HPLC yielded a relatively homogenous fraction containing bovine LPS-binding protein activity, whereas purification of rabbit LBP does not require this added step (21).

The LPS-binding and biological functions of the HPLC purified LPS-binding protein requires additional characterization, and the available methodology should now make it possible to upscale LPS-binding protein purification in order to carry out further necessary studies. Even though the functions of bovine LPS-binding at the cellular level needs to be elucidated, our findings have notable implications for researchers utilizing bovine sera or other biological fluids derived from bovine sources, and for those researchers interested in gram-negative bacterial diseases in the bovine species. Identification of bovine LPS-binding protein is an important step in characterizing the cellular effects of bovine LPS-binding protein both in vitro and in vivo. Our findings may be of special interest to those researchers who have used bovine serum in studies and have observed significant potentiation of the biologic effects of LPS, as it appears that an analog of human and rabbit LBP is also present in bovine serum. Homology between human and bovine LPS-binding protein may provide future

opportunity for interesting comparative studies of the molecular structure and mechanism of action of LBP.

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APPENDIX

FIG. 1 (A) Fractionation of bovine serum using Bio-Rex 70 resin. A representative chromatographic elution profile. Bound bovine serum was eluted using 41 mM, 220 mM, a linear gradient (220-500 mM NaCl) and 1 M NaCl in 50 mM sodium phosphate, pH 7.3,containing 2 mM EDTA. Arrows indicate protein peaks obtained with solvents used, as monitored by absorbance at 280 nm. Five protein peaks or pools (1-5) were obtained using Bio-Rex 70 columns.

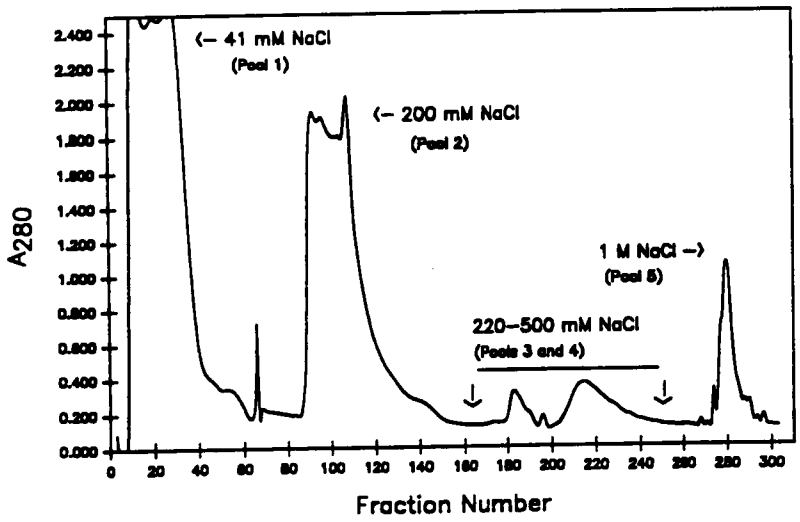


FIG. 1 (B) Silver stained SDS-PAGE of protein peaks obtained from Bio-Rex 70 column. Equal amounts of protein from pools 1-5 were loaded on to a 10% SDS-polyacrylamide gel; pool 1 (lane 1), pool 2 (lane 2), pool 3 (lane 3), pool 4 (lane 4) and pool 5 (lane 5). Molecular masses (kDa) of prestained protein standards are given to the right. A heterogenous mixture of proteins is seen in each lane.

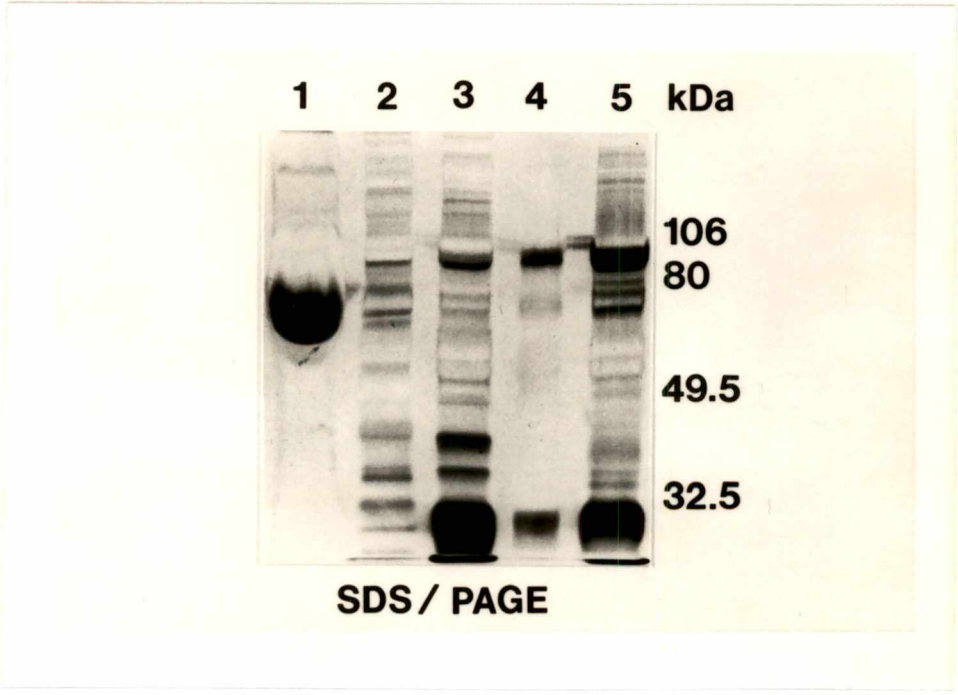


FIG. 2. Photoaffinity labeling of protein pools eluted from Bio-Rex 70 column using ^{125}I-ASD-LPS. 60 μg of protein from pools 1-5 as seen in Fig. 1A and BSA (control) was incubated with 0.5 μCi ^{125}I-ASD-LPS, (0.2 μg LPS) and photoactivated; the incubation mixture was electrophoresed on a 10% SDS-polyacrylamide gel. Following autoradiography, radiolabeled bands were seen in some of the lanes. BSA is in lane 1; pool 1 is lane 2; pool 2 is in lane 3; pool 3 is in lanes 4 and 5; pool 4 is in lanes 6 and 7, pool 5 is in lane 9, and ^{125}I-ASD-LPS alone is in lane 8. Molecular mass (kDa) of protein standards are indicated to the left. Pool 2 (lane 3) shows a relatively intense 60 kDa band.

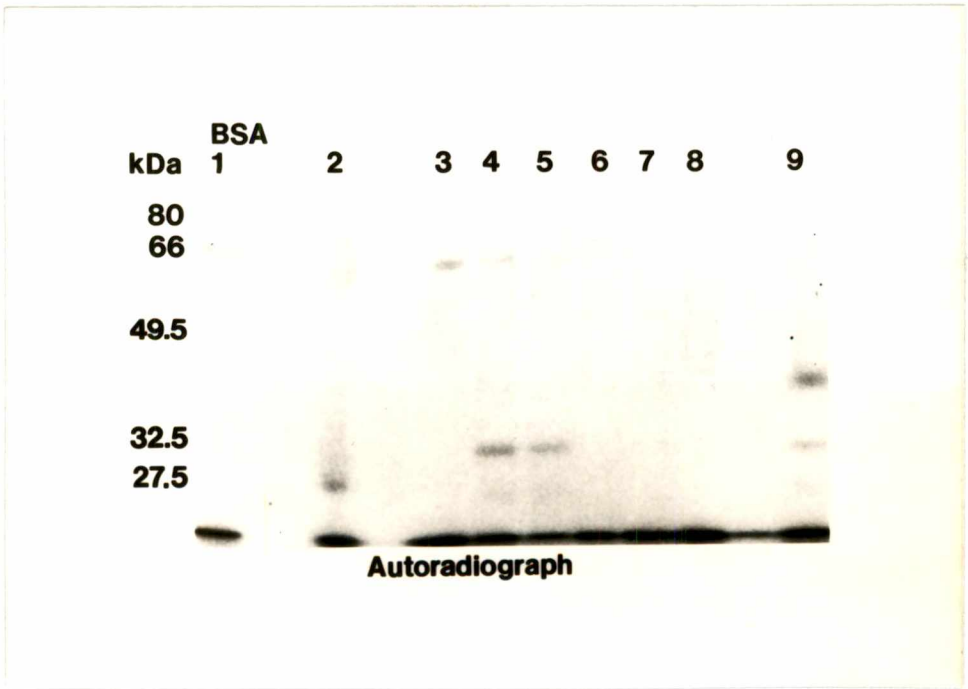


FIG. 3. Binding of ^{125}I-ASD-LPS to varying amounts of protein from pool 2 obtained from Bio-Rex 70 column. A fixed amount (0.5 μgs) of photoaffinity probe (^{125}I-ASD-LPS) was incubated with various amounts of protein from peak 2 and photoactivated. The incubation mixture was then electrophoresed on a 10% SDS-polyacrylamide gel and autoradiography subsequently performed. The BSA control (60 μg) is in lane 1; lanes 2-6 contain 72, 36, 24, 12 and 6 μgs of pool 2. Molecular mass (kDa) of prestained protein standards are shown to the right. An arrow indicates the 60 kDa LBP of interest. Only the 60 kDa protein band appears with the lowest amount of protein. Upon increasing the amount of protein, two other bands of different intensity are observed.

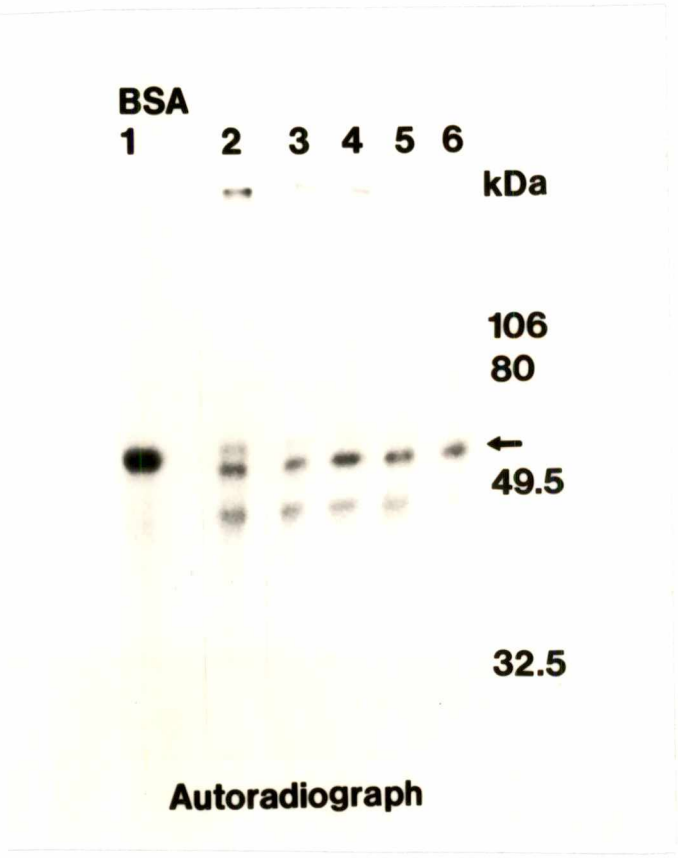


FIG. 4. Competition of unlabeled and radioiodinated LPS (^{125}I-ASD-LPS) binding to proteins in pool 2 eluted from Bio-Rex 70 column. A fixed amount of ^{125}I-ASD-LPS (0.5 μgs LPS) and 60 μgs of protein was incubated in the absence and presence of various amounts of unlabeled (*E.coli* 0111.4) LPS and photoactivated. The incubation mixture was electrophoresed on a 10% SDS-polyacrylamide gel and autoradiography was performed. Lanes 1 and 2 have BSA (control) bound to ^{125}I-ASD-LPS in the presence (lane 1) or absence (lane 2) of 10 μgs unlabeled LPS. Lanes 3-7 have sample from pool 2. Binding of the photoaffinity probe to pool 2 in the absence of competitor is seen in lane 3, and in the presence of 10 μg (lane 4), 2 μg (lane 5), 1 μg (lane 6), and 0.5 μg (lane 7) unlabeled LPS. An arrow indicates the 60 kDa LBP of interest. Molecular mass (kDa) markers of prestained protein standards are seen to the right. Binding of ^{125}I-labeled LPS to the 60 kDa protein appears to be inhibited in the presence of a 20-fold excess of unlabeled LPS.

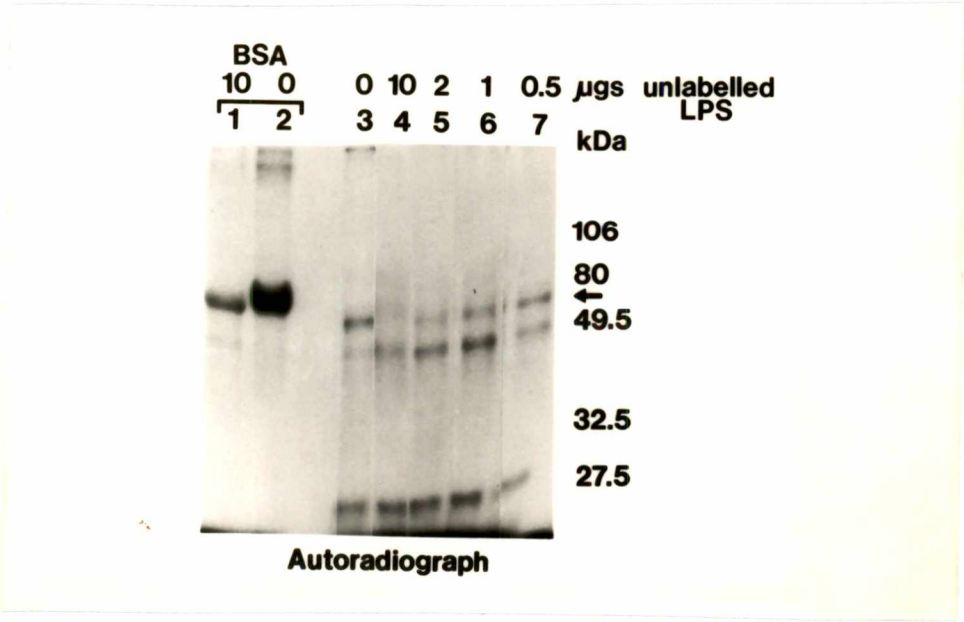


FIG. 5. (A) A Coomassie brilliant blue-stained SDS-PAGE gel of pool 2 and pool 5 eluted from the Bio-Rex 70 column. Lane 1 has pool 2, lane 2 has pool 5, and BSA and prestained molecular mass markers are in lanes 3 and 4 respectively. Molecular mass (kDa) of protein standards are seen to the right. An arrow shows the 60 kDa protein band is present only in pool 2 (lane 1).

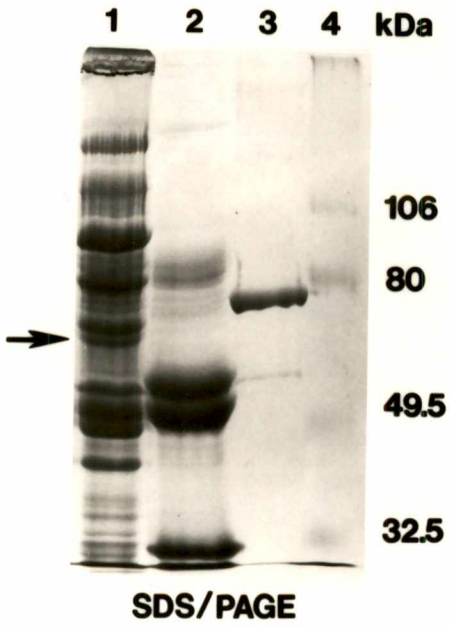


FIG. 5. (B) N-terminal sequence of bovine LBP. The 22-residue N-terminal sequence of bovine LBP (the 60 kDa band) is shown. Comparison to human LBP (17) indicated 77% sequence homology, 68% sequence homology to rabbit LBP (21), and 50% sequence homology to human BPI (22).

1	Bovine LBP:	A N P G L V V R I I D Q G L E Y V A Q E E L
2	Human LBP:	A N P G L V A R I I D K G L Q Y A A Q E Q L
3	Rabbit LBP:	T N P G L I T R I I D K G L E Y A A R E Q L
4	Mouse LBP:	T N P G L V T R I I
5	Human BPI:	V N P G V V V R I S Q K G L D Y A S Q Q Q T
6	Bovine BPI:	T N P G I V A R I I Q K G L D Y A C Q Q G V

FIG. 6. Purification of LBP using HPLC anion exchange Mono-Q HR 5/5 column. Elution profile of purification of LPS-binding protein utilizing HPLC. An aliquot of pool 2 (Fig. 1A) was injected onto a Mono-Q HR 5/5 column. Protein was eluted using a gradient (0-50 and 50-333 mM) of ammonium sulfate in 20 mM diethanolamine buffer, pH 8.3. The dashed line indicates the gradient of ammonium sulfate; the solid line indicates elution of material absorbing at 280 nm.

(A) Representative elution profile of the first run of pool 2 on the Mono-Q column. Two closely eluted protein peaks with retention time of 34.82 (fraction 69) and 35.27 min (fraction 70) are seen.

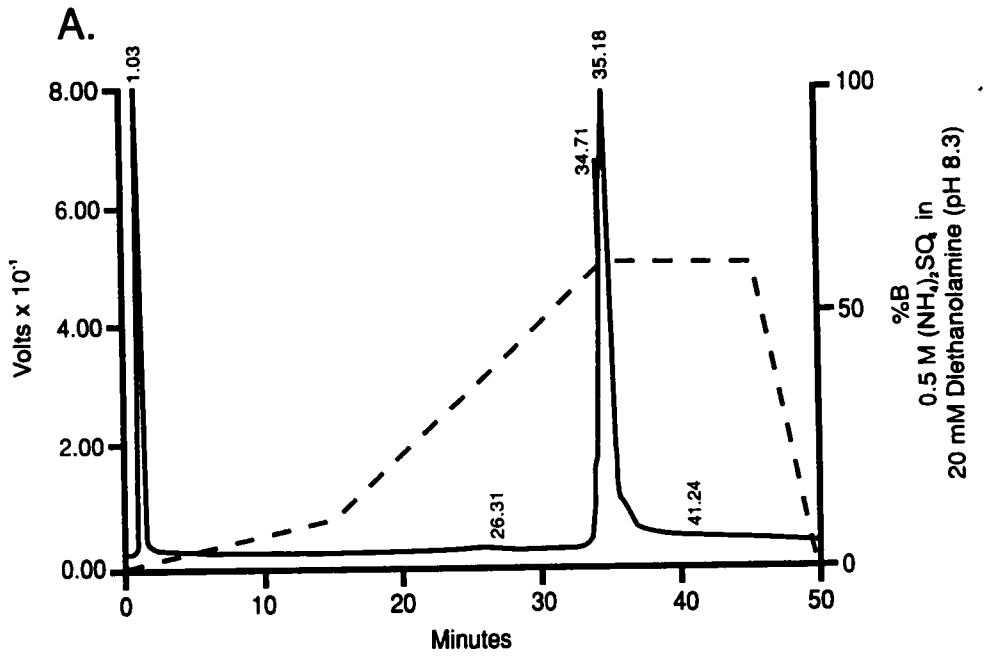


FIG. 6. Purification of LBP using HPLC anion exchange Mono-Q HR 5/5 column. Elution profile of purification of LPS-binding protein utilizing HPLC. An aliquot of pool 2 (Fig. 1A) was injected onto a Mono-Q HR 5/5 column. Protein was eluted using a gradient (0-50 and 50-333 mM) of ammonium sulfate in 20 mM diethanolamine buffer, pH 8.3. The dashed line indicates the gradient of ammonium sulfate; the solid line indicates elution of material absorbing at 280 nm.

(B) Rechromatography of fraction 70 using the same gradient as used in the previous run yields further purification.

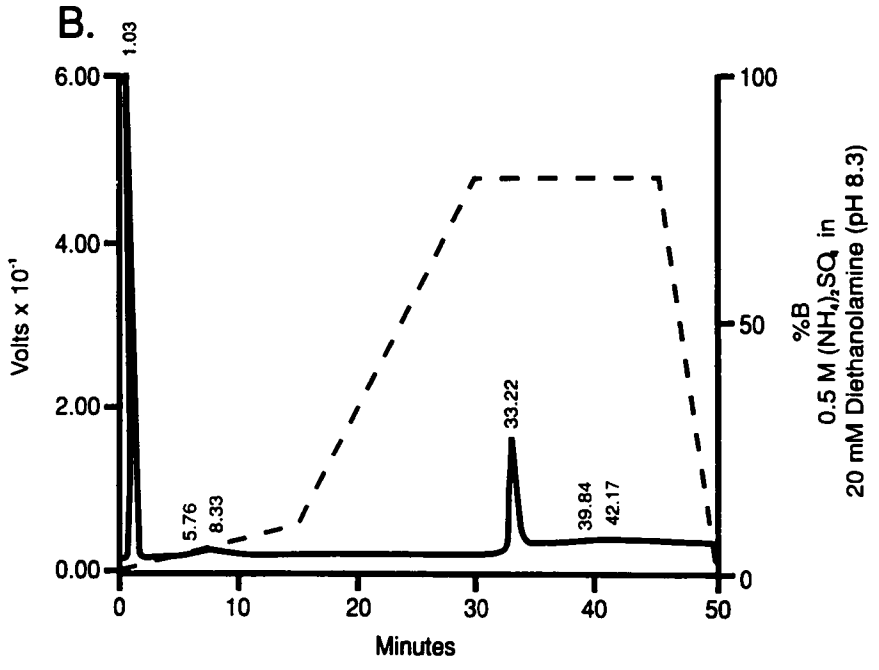


FIG. 6 (C) SDS-PAGE of protein eluted from HPLC. A 10% SDS-polyacrylamide mini-gel was run to analyze protein fractions and silver-staining was used to visualize protein eluted from the first and second run on the Mono-Q column. Fractions 67 and 68 from the second run on the Mono-Q column (Fig. 6B) are in lanes 1 and 2. Fractions 69 and 70 from the first run, (Fig. 6A) are in lanes 3 and 4. Molecular mass (kDa) of prestained protein standards are shown to the left. It is seen that rechromatography under the above conditions yields a relatively cleaner protein fraction (lane 2).

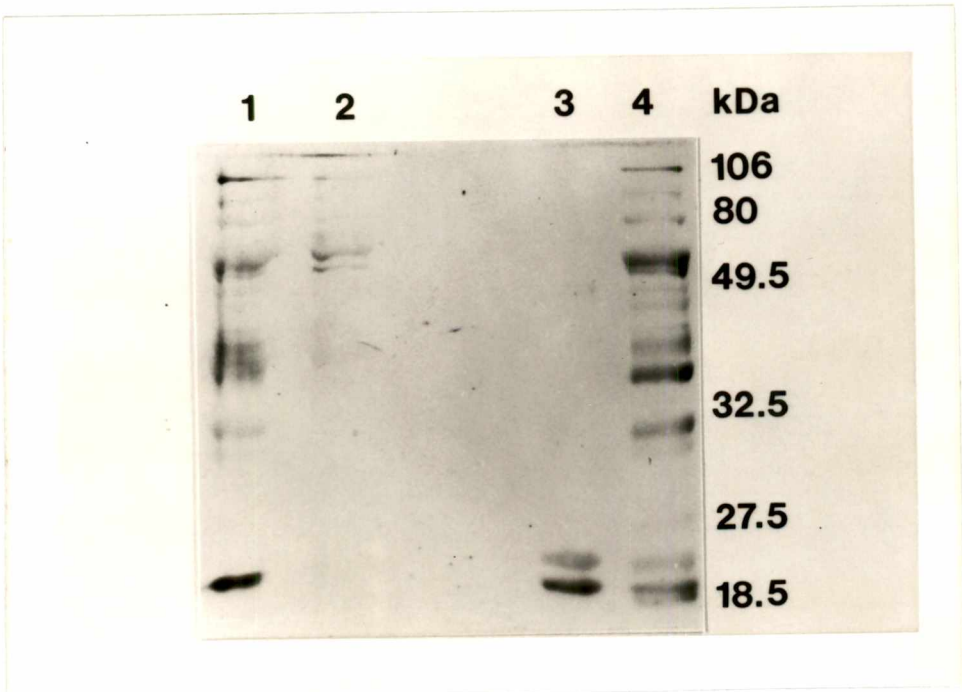


FIG. 7. Autoradiograph of cross-linked proteins from pool two (Fig. 1A). An aliquot of the protein was incubated with ^{125}I-ASD-LPS, photoactivated and chromatographed twice on the Mono-Q column as in 6 (A and B). Following rechromatography, fraction of interest was collected, concentrated using a speed vacuum and electrophoresed on a 10% SDS-polyacrylamide gel. Lanes 1 and 2 show fractions 67 and 68 eluted from the HPLC. Molecular mass (kDa) of prestained protein standards is shown to the left. As seen from the autoradiograph fractions 67 and 68 are indeed LPS-binding proteins of apparently 70 kDa.

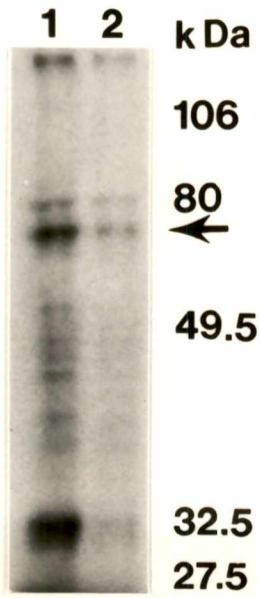


FIG. 8 (A). Purification of LBP using HPLC anion exchange Mono-Q HR 5/5 column. Elution profile of purification of LPS-binding protein utilizing HPLC. An aliquot of pool 2 (FIG. 1A) was injected onto a Mono-Q HR 5/5 column. Protein was eluted using a gradient (0-50 and 50-333 mM) of ammonium sulfate in 20 mM diethanolamine buffer, pH 8.3. The dashed line indicates the gradient of ammonium sulfate; the solid line indicates elution of material absorbing at 280 nm.

(A) Representative elution profile of pool 2 run on the Mono-Q column. Two major and well separated peaks are seen with retention times of 22.13 and 38.55 minutes.

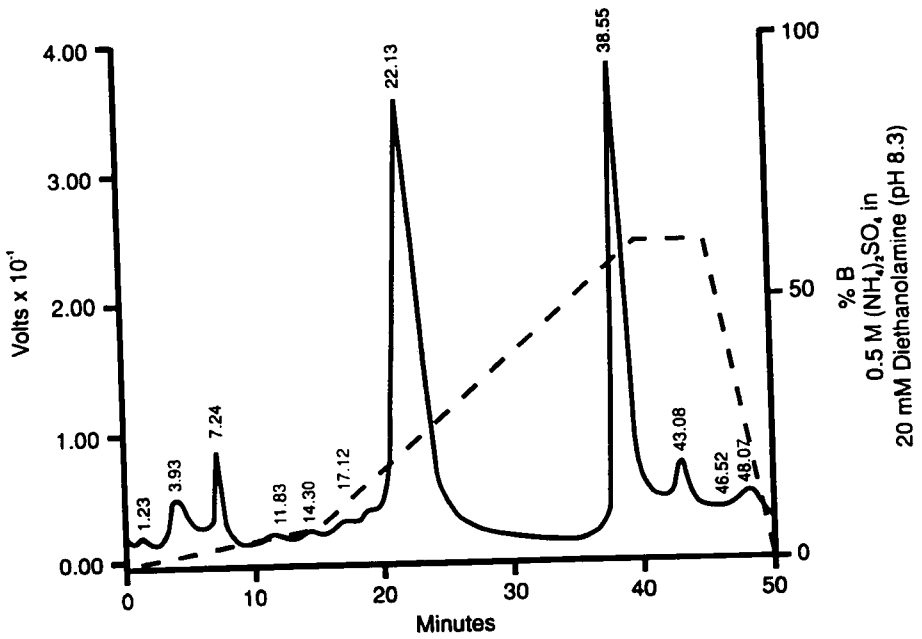
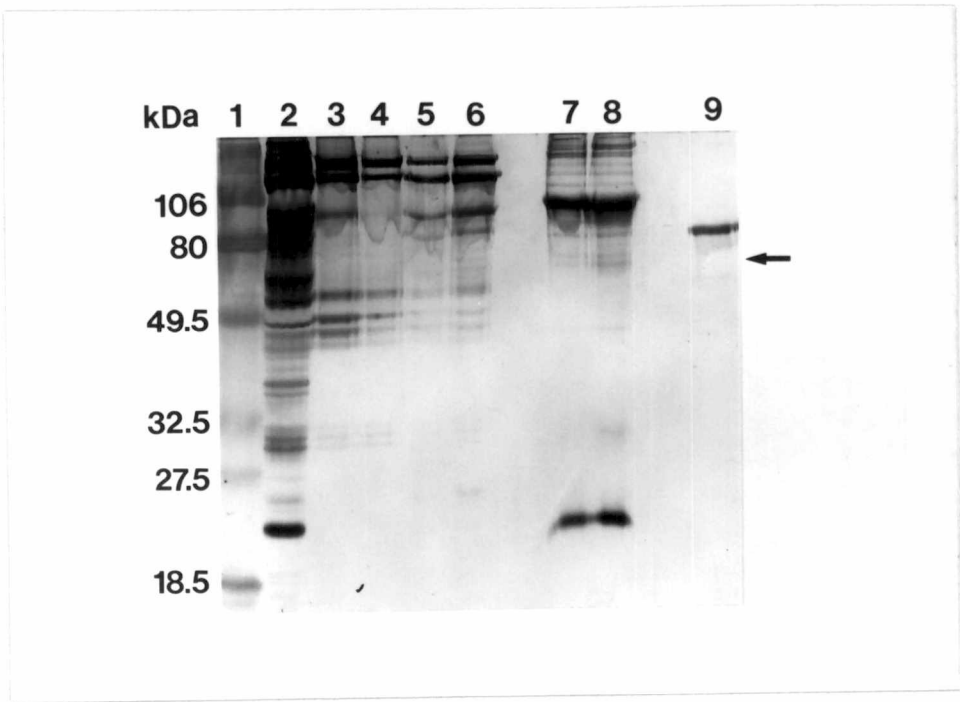


FIG. 8 (B) SDS-PAGE of protein eluted from HPLC. A 10% SDS-polyacrylamide gel was run to analyze protein fractions and silver staining was used to visualize protein eluted from the Mono-Q column. Lanes 2-4 show fractions collected around and at 22.13 minute peak and lanes 5-7 show fractions collected around and at the 38.55 minutes peak and are referred to pools 4-6. Prestained molecular mass markers (kDa) are seen in lane 1. It is seen that a 60 kDa protein band (arrow) is seen only in lanes 7 and 8.



PART 4

GENERATION OF POLYCLONAL ANTIBODIES AGAINST BOVINE 60 kDa LPS-BINDING PROTEIN AND BIOLOGICAL ACTION OF PROTEIN FRACTIONS CONTAINING THE 60 kDa LPS-BINDING PROTEIN

ABSTRACT

Lipopolysaccharide-binding protein (LBP), initially identified in rabbit serum, has now been identifed in murine, human and adult bovine serum. LBP is an immunoregulatory protein and it has been shown to bind to bacterial lipopolysaccharides (LPSs) with a high affinity. Upon this binding, the LPS-LBP complex has been shown to bind to a membrane associated receptor called CD14. This interaction, has been shown to make cells such as monocytes and macrophages exquisitely sensitive to very low concentrations of LPS. In this study, the effect of LPS in the presence of normal adult bovine serum (NBS), protein fractions obtained upon fractionation of normal adult bovine serum using ion exchange chromatography and further purification, using HPLC, of the a specific LPS-binding protein of 60 kDa on tissue factor expression by bovine alveolar macrophges has been shown. LPS, by itself at 1 ng/ml failed to induce measurable tissue factor expression by macrophages, but the presence of NBS, "pool 2" and HPLC purified fractions markedly augumented the action of LPS. In combination with 1 ng/ml LPS, 800 μg/ml of NBS, 20 μg/ml of "pool 2", and only 0.5-1 μg/ml of HPLC purifified protein was required. These results indicate that the first step of protein purification concentrated the activity that enhances LPS action by 40 fold, whereas HPLC purification further concentration this protein by 800-1600 fold compared to the normal adult bovine serum. The stimulatory effect of LPS in the presence of protein fractions containing the 60

kDa LPS-binding protein, was abrogated when monoclonal antibodies (60 bd, My4; 10 and 20 μg/ml) directed against CD14 were used. This showed that the augumentary effect of LPS-LBP complex was CD14 dependent. Performing the same assays in presence of heat-treated proteins and polymyxin B (10 μg/ml) provided evidence that the protein fractions contained an heat labile and lipid A binding protein that was responsible for potentiated LPS effects.

CHAPTER 1

INTRODUCTION

Lipopolysaccharides (LPSs) are major components of the outer membrane of gram-negative bacteria. These molecules are potent inflammatory agents and gram-negative bacterial infections have been known to cause pleiotropic effects in the host immune system (1, 12, 26, 36, 41, 48, 54). Most of the pathogenesis caused due to these infections is related to the lipopolysaccharide molecule interacting with host cells either directly or by binding to LPS-binding proteins (2, 13, 35, 47, 58, 69, 70). Thus stimulated various host cells are either directly or via potentially lethal mediators of septic shock released due to such interactions (5, 47, 54, 60, 65, 69).

Cells of the monocytic lineage play a pivotal role in determining the host response during endotoxemia (47, 63, 69). These cells have been shown to be exquisitely sensitive to very low, in the nanogram to picogram range, concentration of LPS. Some of the effects that are altered due to LPS-induced stimulation of macrophages include phenotypic and functional changes (19, 43, 45, 54). Several studies have shown that an intricate relationship or an interplay exists between cytokines such as tumor necrosis factor (TNF- α), interleukin-1- α (IL-1 α), IL-6, IL-8, NO, eicosanoids and tissue factor released by monocytes/macrophages (47, 58, 69). Interaction of LPS with monocytes/ macrophages could be beneficial to the host, because these cells remove LPS from circulation (47, 48) and synthesize

cytokines that can be beneficial for the host defense. However, an excessive production of these mediators can result in adverse consequences (47, 48, 54).

The lung plays a crucial role in hemostasis. The unregulated deposition of fibrin has been reported to be the cause of the pathogenesis of a wide variety of inflammatory lung diseases (6, 11, 23, 26, 27, 34, 36, 54). The clotting system has been divided into the intrinsic and the extrinsic pathways. Both these pathways merge to form a final common pathway that leads to conversion of fibrinogen to fibrin formation (54). Even though either of the two pathways, that is the intrinsic or the extrinsic pathway, can initiate the fibrin formation, only the extrinsic pathway involves a "tissue factor" in addition to blood components (54). This factor is found in tissues rather than in blood and therefore it is referred to as "tissue factor" (54).

The procoagulant activity (PCA) of monocytes and macrophages has been known to be induced in response to a variety of physiological and pathological mediators such as LPS, cytokines, immune complexes and activated complement (3, 8, 9, 10, 19, 25, 32, 39, 48, 66, 68, 76). The PCA effector molecule is tissue factor (TF) (54). Tissue factor, also called thromboplastin (factor III), is a cell surface, transmembrane glycoprotein which can initiate blood clotting in the presence of calcium, phospholipids and other coagulation factors (54). Tissue factor functions as a high affinity receptor for coagulation factors VII and VIIa in the activation of factors IX and X (54). Activation of the extrinsic pathway of coagulation by tissue factor is one of the ways that leads to deposition of fibrin in

the pulmonary alveolus (54). Under normal conditions accumulation of alveolar fibrin is regulated through the activation of the serine protease urokinase plasminogen activator (uPA) produced more actively by pulmonary alveolar macrophages as compared to neutrophils (54). Plasminogen activator converts the inactive precursor plasminogen to the active protease plasmin (54). Plasmin functions in a manner analogous to trypsin and thrombin. That is it cleaves the lysine-arginine bonds. Plasmin mediates the physiologically important process of fibrinolysis by cleaving fibrin to fibrin degradation products (FDP's). However, when activation of plasminogen is inhibited by plasminogen activator inhibitors, also secreted by macrophages, fibrosis occurs (54). This leads to many types of acute lung injury in humans and in animals (26, 27, 54). Thus, leukocytes such as macrophages, play an important role in the coordinated expression of coagulation and fibrinolytic functions.

Adam et. al. (1) have shown that neonatal calves, upon administration of LPS, elicit circulating TNF-α. *In vitro* studies done by the same researchers has shown that LPS stimulated peripheral bovine monocytes and macrophages release TNF-α (2). Studies done by Car et. al. (9, 10) have shown that stimulation of bovine alveolar macrophages by LPS leads to expression of tissue factor activity. However, LPS-induced release of TNF-α from monocytes and macrophages (2) and the maximal expression of tissue factor by LPS stimulated bovine alveolar macrophages have been shown to occur at much higher concentrations of LPS (microgram/ml) than those that are physiologically relevant.

Recent studies have identified and characterized a LPS-binding protein (LBP), from rabbit (57, 58) murine (21, 58) and human (51, 58) serum that plays a critical role in the early detection of LPS during a bacterial infection. Several studies have shown LBP to shift the threshold for LPS-induced macrophage activation (13, 20, 34, 35, 52, 58, 62). LPS in serum has been shown to form high affinity complexes with LBP and LPS-LBP complex has been shown to bind monocytes and macrophages through a membrane associated receptor called mCD14 (71, 75). This interaction activates the cell through mechanisms not yet fully elucidated, however, activation of tyrosine kinase has been shown to be involved (22, 67, 72). To date, published literature does not indicate that research has been done to identify the presence of a similar bovine LPS-binding protein that could infuence and shift the threshold of LPS-induced macrophage activation. Previous studies (28, Part III), done earlier in this research project have identified purified and to some extent characterized a 60 kDa LPS-binding protein that has significant homology in the first 20 amino acids of the N-terminal sequence homology with the rabbit (57, 58), murine (58) and the human (51, 58) LBP. In this section, the biological activity of protein fractions containing the bovine 60 kDa LPS-binding protein will be studied. Specifically expression of tissue factor will be observed by bovine alveolar macrophages in the presence of these protein fractions and LPS. A colorimetric assay based upon the digestion of a substrate by activated Factor Xa to assess tissue factor expression will be used. The rationale for the procoagulant assays is that if functional bovine LBP is present

in the concentrated chromatographic fractions, macrophages should express procoagulant activity (as tissue factor) at much lower concentrations of LPS when fractions containing the 60 kDa LPS-binding protein is present (as LPS-LBP complexes).

CHAPTER 2

MATERIALS AND METHODS

Materials

LPS from *E. coli* 0111:B4 was purchased from List Biological Laboratories (Campbell, CA, USA). Bovine serum albumin (BSA), polymyxin B and LPS derived from *E. coli* (serotype 055:B5) used in this study were purchased from Sigma Chemical Co. (St. Louis, MO, USA) The *LPS from E. coli* 055:B5 contained 11,00endotoxin units/microgram as determined with a chromogenic *Limulus* assay (QCL-1000, Whittaker bioproducts, Inc., Walkersville, MD). Sulfosuccinimidyl-2-(p-azidosalicyl-amido)1,3'-dithiopropionate (SASD) was from Pierce Chemicals (Rockford, IL, USA). Bio-Rex 70 resin, prestained molecular weight standards, nitrocellulose membrane (0.2 μm) and other materials for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) were obtained from Bio-Rad Laboratories (Richmond, CA, USA). Sephadex G-50 and Protein-A Sepharose was purchased from LKB Pharmacia (Piscataway, NJ, USA). The ultrafiltration cell and YM10 membranes were obtained from Amicon Corp., (Danvers, MA, USA). Na ^{125}I was from ICN (Lisle, IL, USA). New Zealand white rabbits were obtained from a local supplier, Fruend's incomplete adjuvant was purchased from Sigma Chemical Co. (St. Louis, MO, USA. DMEM was purchased from Whittaker BioProducts, Walkersville, MD. and Hank's Balanced Salt Solution (HBSS) was from GIBCO Laboratories, (Grand Island, NY). 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 Travenaol Labs, Inc., Glendale, CA. My4 is an anti-human CD14 murine IgG_{2b} monoclonal antibody, and was purchased from Coulter Immunology (Hialeah, FL). The murine anti-human CD14 antibody 60bd (IgG_1, 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. mAb 60bd was purified with an ImmunoPure (G) IgG Purification Kit (Pierce, Rockford, IL). Murine control antibodies (purified IgG_{2b} and IgG_1) were from Organon Teknika Corp., (Malvern, PA.).

Serum Samples

Sera were obtained by jugular venipuncture from healthy adult cows, pooled, and used for these studies. Animals serving as blood donors received the highest level of care, and all protocol were reviewed and approved by the University Animal Care and Concerns Committee. Whole blood was allowed to clot for 1-2 h at 37°C, centrifuged (20 min, 1200 g, 22°C), the clots compressed and similarly re-centrifuged (30 min, 2200 g, 4°C). Collected serum was then similarly recentrifuged to render it platelet-poor, and finally frozen without preservative (-70°C) until use. Sera used in our studies contained 7.9 grams/dL of total protein.

Purification of LPS-binding Protein

Bovine serum was first fractionated using Bio-Rex 70 column as previously published (57). Protein eluted from the column was monitored at 280 nm, samples were pooled into five fractions, and were dialyzed overnight against 5 mM Hepes, pH 7.3 (4 ℃). The fractions were next concentrated approximately 10 fold using an Amicon (YM 10 membrane) ultrafiltration cell. The concentrated protein was then aliquotted into 1 ml fractions and lyophilized using a speed vacuum (Jouan, RC1010, -60 °C); concentrated protein was stored at -70 °C until further use. These experimental methods have been repeated six times with similar results in our laboratory, using various pooled lots of serum obtained from several animals.

The second chromatographic step utilized HPLC (Waters), was performed according to the procedure described by Tobias et al. (57), with some modifications. A Mono-Q HR 5/5 column was used as the adsorbent, and the flow rate throughout the HPLC step was 1 ml/min. Briefly, the Mono-Q column was equilibrated with 2 mM diethanolamine buffer, pH 8.3. Injection of the sample was followed immediately by a 15 ml gradient of 0-50 mM ammonium sulfate in 20 mM diethanolamine, pH 8.3. The gradient was then steepened, going in 20 ml from 50 to 333 mM ammonium sulfate in the same buffer. Finally the column was washed with 10 ml of 333 mM ammonium sulfate in 20 mM diethanolamine, pH 8.3 for 10 min. Elution of protein was monitored by absorbance at 280 nm and two fractions of 0.5 ml each were collected per min.

Fractions of interest were first concentrated using a speed vacuum, then resuspended in 20 mM diethanolamine buffer, pH 8.3,and under similar (with the exception of the second, 50 to 333 mM, linear gradient which was accomplished in 15 rather than 20 min.) conditions to those above were rechromatographed on the Mono-Q column. In some instances, protein obtained from pool 2 (eluted using 220 mM NaCl) of Bio-Rex 70 column chromatography was photoaffinity labeled using ^{125}I-ASD-LPS prior to chromatography on HPLC (see next method)

In some instances, Mono Q column was washed with a lesser (20 ml instead of 40 ml or more) amount of 20 mM diethanolamine prior injection of protein.

Photoaffinity labeling

E. coli 0111:B4 LPS was first sonicated, then coupled to SASD as described previously (61). The resulting LPS-ASD complex was radiolabeled with Na ^{125}I using Chloramine T as described (24, 25). The product typically had a specific activity of 6 x 10^9cpm/mg LPS. ^{125}I-ASD-LPS (0.5 μCi) containing 0.2 μg of LPS was then incubated with protein (60 μgs) from Bio-Rex 70 chromatography pools in borosilicate glass tubes for 10 min at 37 °C. Control incubations included BSA (60 μgs), and the others included radiolabeled LPS alone. Cross-linking of the ligand to the protein was then accomplished by photolysis for 10 min at room temperature by using short-wave UV irradiation (Hoefer Scientific Instruments, CA., UVC 1000 cross-linker, maximal emission at 254 nm). After cross-linking, some samples were concentrated using a speed vacuum.

Generation of Polyclonal Antibodies to Bovine LPS-binding protein

Rabbit anti-bovine LPS-binding protein polyclonal antibodies were generated using standard immunological procedures (33). New Zealand White rabbits were injected at multiple subcutaneous sites with isolated bovine LPS-binding protein (approx. 0.1 mg) from SDS-PAGE gel slices that has been combined with Freund's incomplete adjuvant. After two weeks, the procedure was repeated using incomplete Freund's adjuvant. Two weeks following the second injections the rabbits were boosted again, and blood was collected from the marginal ear vein, the serum harvested, and was assayed for reactivity with LBP by, *in vitro* bioassays and by western blot. Administration of additional periodic booster injections was continued. Bovine anti-LPS-binding protein antibodies were concentrated using a protein A affinity column (Protein A Sepharose CL-4B, Pharmacia). The protein concentration was determined using the Bradford method.

Purification of Polyclonal Antibodies Using Protein-A Column IgG from serum of rabbits immunized with the bovine 60 kDa LPS-binding protein was purified by using protein-A column. Protein-A column packing and IgG elution was carried out essentially by following the protocols by Margulies (33).

SDS-PAGE and Western Blot (Immunoblot)

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on a 10% acrylamide slab gel according to the method of Laemmli (29)

and bands were visualized with Coomassie blue. Before electrophoresis, samples were boiled for 2 minutes in sample buffer with 5% mercaptoethanol, 10%glycerol, and 2% SDS. Protein transfer to a nitrocellulose (Schleicher and Schuell) (59) was performed with a semidry electrophoretic transfer cell (Trans-Blot SD; Bio-Rad Laboratories, Richmond, CA) and incubated with polyclonal rabbit anti-LPS-binding protein (60 kDa) at a 1:1000 ratio. Protein bands were visualized by using an Immunoblot kit (Bio-Rad) that utilized alkaline phosphatase method with 4-chloro-1-naphthol as the substrate.

Immunoprecipitation and SDS-PAGE

The 60 kDa LPS-binding protein containing bovine serum fraction (pool 2) obtained using Bio-Rex 70 resin was used for immunoprecipitation studies. Prior to immunoprecipitation, pool 2 proteins were photoaffinity labeled using ^{125}I-ASD-LPS. Next, varying amounts of polyclonal antibodies were added to the test tubes containing reaction mixtures and incubation was allowed to take place with gentle shaking overnight at 4°C. Controls included preimmune rabbit IgG, commercial IgG and BSA. In order to precipitate the immunocomplex and isolate it, the above reaction mixture was incubated with protein-A Sepharose beads (Pharmacia) for 30 minutes at 4°C and centrifuged in microfuge tubes. The supernatant was separated from the pellet and run on a 10% SDS-PAGE. The protein bands were visualized by Coomassie blue R250 or by autoradiography. The relative mobilities of proteins were determined using prestained low-range

molecular mass protein standards and BSA. When autoradiography was performed, gels were dried on a Slab gel drier (Hoefer) and Kodak X-Omat XAR-5 film was used with the aid of a Dupont Cronex Lighting Plus Intensifier Screen (X-Ray of Georgia, Norcross, GA, USA). Gels were kept at -70 °C until (5-24 hr) the desired intensity was obtained.

Collection of Bovine Alveolar Macrophages

Alveolar macrophages were collected from 1-3 month-old Holstein-Frisian calves by bronchopulmonary lavage as has been described (30). Lavage fluid with cells was first filtered through sterile gauze, and the cells were pelleted by centrifugation (200 g for 10 minutes), and were resuspended and washed twice in sterile, pyrogen-free saline before final resuspension in Hank's balanced salt solution (HBSS) containing divalent cations and HEPES (20 mM, pH 7.4) and kept on ice until assayed. The percentage of macrophages was determined by Wighte-Giemsa staining was greater than 92%, with the remaining cells neutrophils, and very small numbers of epithelial cells, eosinophils, or lymphocytes. And the viability determined by trypan blue dye exclusion was greater than 92%.

Procoagulant Activity Assay for Bovine Alveolar Macrophages Stimulated with LPS/LPS-LPS-binding protein(s)

Tissue factor expression on the macrophages was quantitated with a colorimetric assay (2) based on disgestion of substrate S-2222 by activated factor X. Macrophages, at 40,000 cells/well, were plated into 96-well flat bottom tissue culture plates and incubated in DMEM, for two hours, containing 20 mM HEPES, 4 mM L-glutamine, and 100 U penicillin G (Gibco) at 37°C and with 5% CO_2. Macrophages were allowed to adhere and washed twice with serum-free and phenol red-free media (DMEM). Cells were then treated to 1 ng/ml LPS *(E. coli* 055:B5) either in the presence or the absence of different concentrations of normal bovine serum, LPS-binding proteins partially purified using the Bio-Rex 70 column chromatography and HPLC (Mono-Q column).

In some assays (see Fig. legends) macrophages were treated with additional reagents. In some cases, **monoclonal antibodies** (mAb) or isotype control murine antibodies were added 30 minutes before addition of protein and LPS. In other experiments, the LPS-binding protein(s), were **heated** (56°C, 60°C or 72°C) prior to the addition to cells. Controls included native LPS-binding proteins. In a couple of assays, the LPS was incubated with 10 μg/ml of **polymyxin B** for at least 40 minutes before it's addition to cells. Controls included LPS that was not incubated with polymyxin B, and cells in the presence of polymyxin B alone. After all reagents were added, macrophages were incubated for 5 hours at 37°C with 5% CO_2 in a humid chamber. At the end of these incubations cells were washed twice

with DMEM, followed the by addition of 100 μl/well of DMEM without phenol red and containing 200 μgs/ml of substrate S-2222 or S-2765 was then added and 1 unit factor VII/ml of a coagulation factor concentrate also containing factors II, IX and X. After 60 min at 37℃, the optical density was determined using a plate reader at a wavelength of 405 nm. Control wells consisted of wells with media alone, with LPS alone, and with macrophages exposed to mormal bovine serum/Bio-Rex 70/HPLC purified protein in the absence of LPS. Each treatment variable was performed in quadruplicate, and the experiments were repeated to confirm results.

Statistical Analysis

Data were analyzed using a proprietary statistical package (Statistical Analysis System SAS, CAR, NC). $P < 0.05$, was considered to be significant.

CHAPTER 3

RESULTS

Western Blot

Specificity of the serum obtained from rabbits immunized with the 60 kDa bovine LPS-binding protein was shown by Western blot analysis of protein fractions containing the 60 kDa LPS-binding protein. Pool 2 obtained by fractionation of bovine serum, on the Bio-Rex 70 resin, and fraction number 67 obtained from run 2 on the Mono Q column were used as protein samples. Lanes 1 and 4 in Fig. 1 show only one intense band of approximately 60 kDa protein. However, in lane 2 which contains BSA, as control, a slight band is seen.

Immunoprecipitation Studies

FIG. 2 (lanes 1-6) shows the autoradiograph of radiolabeled supernatants obtained from the immunoprecipitation experiment. Lanes 1 and 2 contain supernatant obtained by utilizing immune IgG, lanes 3 and 4 utilizing preimmune serum, lanes 5 and 6 contain supernatant obtained using commercial rabbit IgG, lane 7 contains pool 2 and lane 8 has BSA. As seen from this figure, only lanes 1, 2 and 8 do not show the 60 kDa band. And, this is because BSA is 66 kDa and in the case of lanes 1 and 2, the 60 kDa LPS-binding protein is immunoprecipitated and since the supernatant has been analyzed, this band is not seen in this autoradiograph. The specificity of the rabbit antiserum is not so clear from this autoradiograph

because it looks like the antiserum recognizes the approximately 64 kDa band to some extent. It is possible that the antiserum recognizes a common determinant in several LPS-binding proteins.

Tissue Factor Expression by LPS Stimulated Macrophages in the Presence of LPS-Binding Protein(s)

Bacterial LPS alone, at a concentration of 1 ng/ml, failed to induce measurable tissue factor expression by macrophages. However, as seen from Fig. 3, the same concentration of LPS in the presence of normal bovine serum, induced tissue factor expression. Expression of tissue factor was measured by a change in the absorbance at 405 nm. Tissue factor expression, by normal bovine serum, was observed even in the absence of LPS. Figure 4 shows that when normal bovine serum that was fractionated using Bio-Rex 70 column chromatography, is added, expression of tissue factor was observed. As seen from the same figure, expression of tissue factor is dependent on the concentration of protein added. In the presence of 1 μg/ml of protein, tissue factor expression is greater than that in the presence of 100 ng/ml of protein. Protein pools 1-4 are obtained by elution of protein from the Bio-Rex 70 column using increasing salt concentrations. Pool 1 elutes with 41 mM NaCl, pools 2a-2c are eluted with 220 mM NaCl. Each pool was collected separately, based upon the protein absorbance at 280 nm. Pool 3 was obtained using an NaCl gradient of 220-500 mM, and pool 4 with 1 M NaCl. Data from this assay shows that only pools 2b, 2c and 3 are able to induce tissue

factor expression in the presence of 1 ng/ml LPS. Macrophages exposed to protein alone did not show tissue factor expression in pools 2 and 3. Pool 1 shows modest tissue factor induction in the presence and absence of LPS.

Since our previous results obtained by SDS-PAGE, autoradiography and *N*-terminal sequencing of the first 20 amino acids showed that it was pool 2 that contained a LPS specific 60 kDa LPS-binding protein (Part III, Chapter 3), this pool was used in further studies. What is interesting in this figure is that pool 3 shows an increased tissue factor expression as compared to pool 2. The reason for this is currently unknown.

Figure 5 shows the expression of tissue factor by LPS stimulated bovine alveolar macrophages increases in the presence of pool 2 in a concentration dependent manner. As seen from Fig. 5, maximal induction of tissue factor expression is observed in the presence of 20 μg/ml of pool 2. The apparent EC_{50} is approximately 3.5 μg/ml. In the absence of LPS, pool 2 alone does not induce much tissue factor expression. A similar assay done with normal bovine serum (Fig. 3) showed that maximal tissue factor expression is achieved with 1600 μg/ml of total protein and EC_{50} of 180 μg/ml in the presence of 1 ng/ml LPS (Fig. 3). However, in this assay, even in the absence of 1 ng/ml LPS, serum alone showed tissue factor expression.

In order to check the biological activity of HPLC purified pool 2 similar assays, to those performed above, were done. Figure 6 shows that once again, tissue factor expression by bovine alveolar macrophages stimulated by LPS is

dependent upon the protein concentrations used. In this case, fractions 70 + 71 were chosen because SDS-PAGE and autoradiography (Part III) had shown these fractions to contain the 60 kDa LPS-binding protein band of interest. As seen from this figure, maximal tissue factor expression is achieved with 1 μg/ml of protein. EC_{50} in this case is 250 ng/ml. In the absence of LPS, protein alone also showed tissue factor expression (data not shown).

Figure 7 shows that, protein obtained by running fraction 70 + 71 over the same Mono Q column for further purification does not diminsh its activity. However, once again macrophages treated with protein alone, that is in the absence of LPS, also shows tissue factor expression (data not shown). We believe that this is possibly due to contamination of the Mono-Q column with LPS. This is supported by data obtained from tissue factor assays done in the presence of protein fractions heat treated prior to tissue factor assays and when polymyxin B is used in addition to LPS (Figs 11 and 12).

In some assays, pool 2 was purified using slightly different HPLC column washing conditions prior to protein injection. This resulted in several protein peaks. Two of the major pools eluted at 22.13 and 38.33 minutes (Part III, Fig. 8(A)). SDS-PAGE had shown the later pool to contain the 60 kDa LPS-binding protein of interest (PART III, Fig. 8(B)). Therefore this pool was collected in two different fractions called fraction 5 and 6. Stimulation of macrophages in the presence of 1 ng/ml LPS and varying concentrations of protein fractions 5 and 6 showed that tissue factor expression was dose dependent in both cases and

maximal activity was obtained at 500 ng/ml (Fig. 8). This showed that fraction 5 and 6 had similar biological activities and for future studies these were combined.

Figure 9 shows that protein pools eluted at 22.13 and 38.55 minutes both have the ability to express tissue factor activity in the presence and absence of 1 ng/ml LPS. However, the 38.55 minute pool has more ability to do so than the other one. Furthermore, it is this pool as mentioned earlier that has the 60 kDa protein of interest. In this assay, maximal tissue factor expression is obtained at 4 μg/ml of protein. EC_{50} for the 38.55 minute pool is approximately 1 μg/ml.

Figure 10 shows that when all the above data is compiled in one graph, the shift in EC_{50} with protein purification is evident.

From all the above data, it is evident that there are several factors in serum that may elevate macrophage tissue factor expression. Some of these are LPS dependent, some are not. This makes construction of a typical purification table difficult and would underestimate the fold purification.

Effects of Heat-Denatured LPS-binding Proteins and Polymyxin B on Tissue Factor Expression

The potentiating effect of components in "pool 2" and HPLC purified protein pools is due to the presence of heat labile LPS-binding protein(s). As seen from Fig. 11, heating, at 72°C, "pool 2c" and HPLC purified protein pools eluted at 22.13 and 38.55 minutes, prior to use in the assay, reduced the expression of tissue factor activity. Greater than 90% decrease was seen in the case of pool 2c

and the 38.55 minute pool. In these assays, 20 μg/ml of pool 2 and 2 μg/ml of HPLC purified protein was used. Heating proteins at other temperatures such at 56℃ and 60℃ also showed a decrease in the expression of tissue factor. Results obtained showed a 39.5% decrease in tissue factor expression when pool 2c was heated at 56℃, 87.4% when pool 2c was heated at 60℃ and greater than 90% when heated at 72℃. Similarly, the HPLC protein pool eluted at 38.55 minutes showed a 90.3%, 66.7% and once again greater than 90% decrease in tissue factor expression when this pool was heated at the above temperatures.

Incubation of LPS with polymyxin B before treating macrophages with LPS, and fractions containing LPS-binding proteins, showed decreased tissue factor expression (Fig. 12). In these assays 1 ng/ml of LPS, 20 μgs/ml of "pool 2" and 2 μg/ml of HPLC purifed protein was used.

Effect of anti-CD14 mAb on Tissue Factor Expression

The effect of anti-CD14 mAbs on tissue factor expression by macrophages exposed to pool 2 (20 μgs/ml) and 1 ng/ml LPS is shown in Figs. 13 and 14. Tissue factor expression was dependent on the concentration of mAbs used (Figs. 13 and 14). As seen from Fig. 13, in the presence of 10 μg/ml of antibody, tissue factor expression is reduced by 40-50% using pool 2 and HPLC purified protein; and 70-80% using 20 μg/ml mAbs (Fig. 14). Similar concentrations of isotype-specific control antibodies did not reduce tissue factor expression. As before, 1 ng/ml of LPS in the absence of protein had no measurable effect in tissue factor expression.

Each data point in all the above assays is the mean of quadriplicate samples. Error bars in some cases are not visibly associated with many data points because the standard error of means of different assays was smaller than the symbol used on the graph.

Statistical analysis done to see whether a significant difference exists between data obtained in the presence and absence of mAb 60 bd and isotype control antibodies yielded a P value of < 0.05 when tissue factor expression was obtained in the absence and presence of 60 bd. However, when the difference between tissue factor expression between the absence and presence of control mAb was compared, a P value > 0.05 was obtained.

CHAPTER 4

DISCUSSION

LPS has been shown to interact with a number of cellular and humoral components (46, 47). Within the host, LPS interacts with cells of the immune system such as neutrophils and monocytes/macrophges either through nonspecific or via specific, receptor mediated, interactions. Plasma components with which LPS interacts include complement proteins, lipoproteins, albumin and recently described LBPs, sCD14 and septin (46, 47, 51, 57, 60, 61, 67, 68). Some of these components either potentiate or abrogate LPS effects thus modulating LPS effects in the host (46, 37, 51, 57, 61).

Recent studies done by Tobias, Ulevitch and co-workers have indicated that LPS binds LBP from murine, rabbit and human serum with a 1:1 stoichiometry and that lipid A portion of LPS is involved in this interaction (51, 57, 61). The LPS-LBP complex has been shown to bind to a membrane associated receptor called CD14 (mCD14) (51, 57, 61 and 69) and this interaction has been shown to potentiate LPS effects. Septin, a plasma protein, recently identifed and characterized by Wright et. al. (68) has also been shown to function in a similar manner, that is via CD14. Septin has also been shown to bind LPS and then interact with the mCD14. Cattle are very sensitive to LPS (1, 2, 15), and, like many other species, are affected by endotoxemia. In response to infused LPS, cattle display similar responses to other species such as LPS-induced cytokine

cascades and resultant pathogenesis (1, 2, 15). Though our, previous studies have indicated the presence of LPS-binding factor(s) in bovine serum (28), to date, component(s) of adult bovine serum that possibly could potentiate LPS effects, in a manner similar to that in other species, have not been isolated and characterized.

Results obtained in the current study extend previous observations by other researchers (51, 57, 61, 69). In previous studies, microgram to nanogram/ml concentrations of purifed LBP from rabbit, murine and human LBP have been shown to enhance response of monocytes/macrophages and neutrophils (13, 20, 33, 34, 51, 57, 61) to LPS effects by 100-1000 fold. Bovine serum, as seen from the results section, has shown to enhance the response of bovine alveolar macrophages to LPS. Furthermore, fractionation and further purification of bovine serum using ion exchange chromatography has shown to concentrate the LPS-binding protein(s) such that much lower concentrations of protein is needed to potentiate LPS effects *in vitro.*

A chromogenic assay that indicated a change in the expression of tissue factor activity was used to demonstrate the biological activity of the 60 kDa bovine LPS-binding protein(s). Protein fractions that contained the LPS-binding activity were indeed capable of inducing tissue factor. That LPS-LBP complexes functioned through the membrane bound CD14 was shown by using anti-human CD14 monoclonal antibodies.

The stimulatory effects of protein fractions that contained the 60 kDa LPS-binding activity were abrogated when LPS was mixed with polymyxin B. Polymyxin B, has previously been shown to bind the lipid A moiety of LPS (39) and to be a potent inhibitor of LPS-induced activation and inhibitor of binding of LPS to monocytes (19, 57, 61). Results obtained using polymyxin B in the tissue factor assay indicate that the protein fractions used in the current assay contain a lipid A-binding protein. Previous studies done using rabbit, murine and human LBP (57, 61) have shown similar results.

Heating protein fractions prior to using them in the tissue factor assay showed a diminished action of the LPS potentiating effects of these protein fractions.

In summary, all the above results indicate that an analog of the previously characterized LBPs also exists in bovine serum. Fractionation of bovine serum LBP using Bio-Rex 70 column and the HPLC yielded purified protein fractions which are very potent, heat labile, polymyxin B inhibitable and potentiate CD14 mediated LPS effects *in vitro*. Since, bovine serum and it's products are widely used and bovine serum contains an LPS-binding protein(s) that potentiates LPS effects, this study sould make researchers more aware of the existence of such an activity in bovine serum.

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APPENDIX

FIG. 1. Western blot analysis of the antiserum to the 60 kDa LPS-binding protein. Polyclonal IgG, to the Bio-Rex 70 column purified 60 kDa LPS-binding protein, was purified using a Protein-A column. Purified IgG was utilized to blot "pool 2" and HPLC purifed pool 2. Lane 1 contains HPLC purified (run 2) "pool 2" proteins, lane 2 has BSA as control, prestained molecular markers are seen in lane 3 and lane 4 contains "pool 2." An intense band of approximately 60 kDa, representing the 60 kDa LPS-binding protein is observed in lanes 1 and 4.

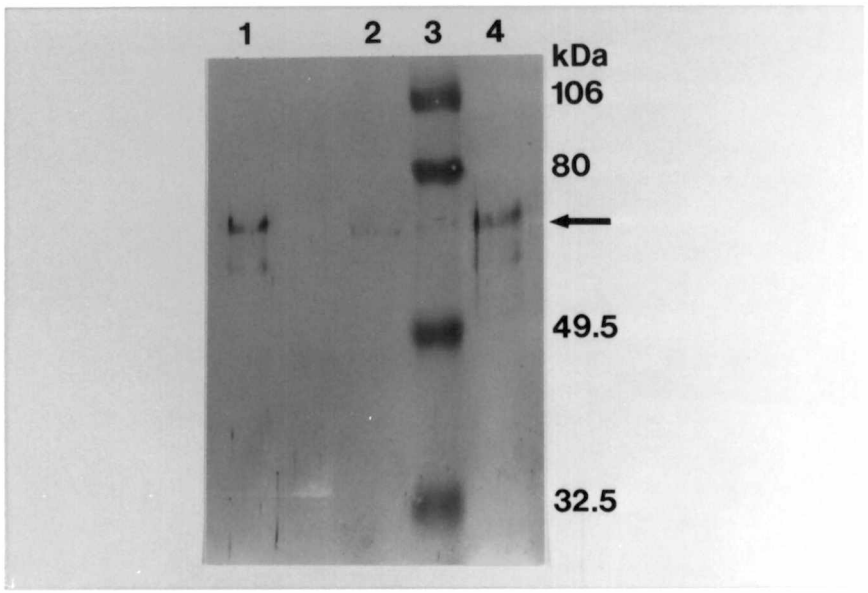


FIG. 2. Autoradiograph of supernatants obtained by immunoprecipitation of LPS-binding protein(s). ^{125}I-ASD-LPS labeled proteins were immunoprecipitated using purified IgG. Supernatants were run on a 10% SDS-PAGE under reducing conditions and autoradiography was performed. Lanes 1 and 2 show supernatants obtained using 25 μgs and 50 μgs of IgG obtained against the bovine 60 kDa LPS-binding protein. Lanes 3 and 4 contain 25 and 50 μgs of rabbit preimmune serum. Lanes 5 and 6 contain supernatants obtained using commercial rabbit IgG, lane 7 has pool 2 alone and lane 8 BSA. Prestained molecular markers are seen to the far left. 60 kDa LPS-binding protein is indicated using an arrow.

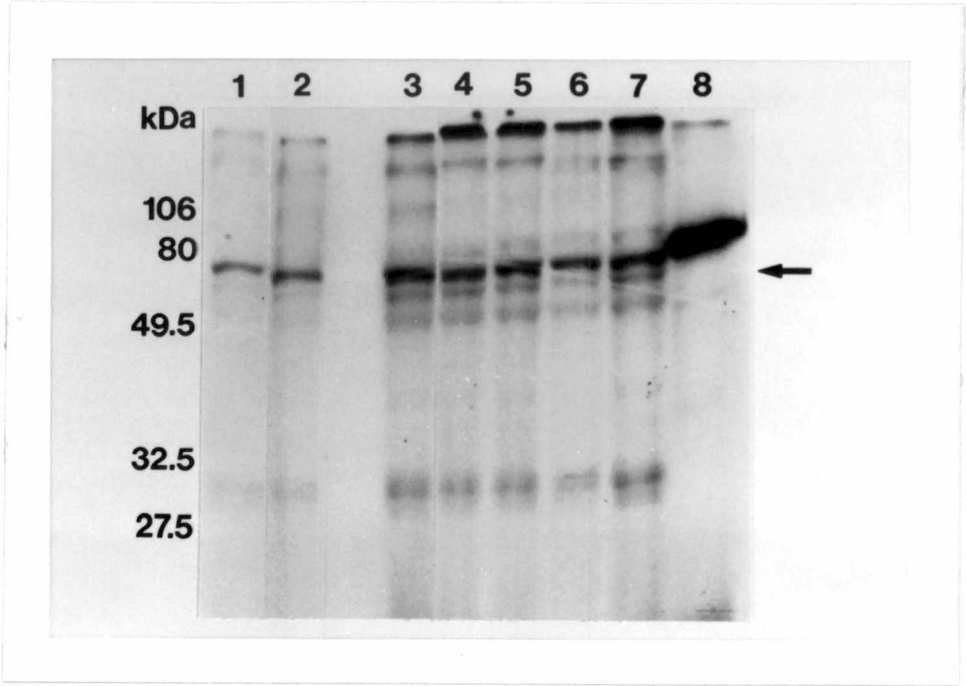


FIG. 3. Effect of dose response of normal adult bovine serum (NBS) on LPS-induced tissue factor expression by bovine alveolar macrophages. Macrophages were exposed to +/- 1 ng/ml LPS from *E. coli* 055:B5. Values are the means +/- S. E. of quadruplicate samples of four different assays; n = 4. Where error bars are not visible, the S. E. was smaller than the size of the symbol used on the graph.

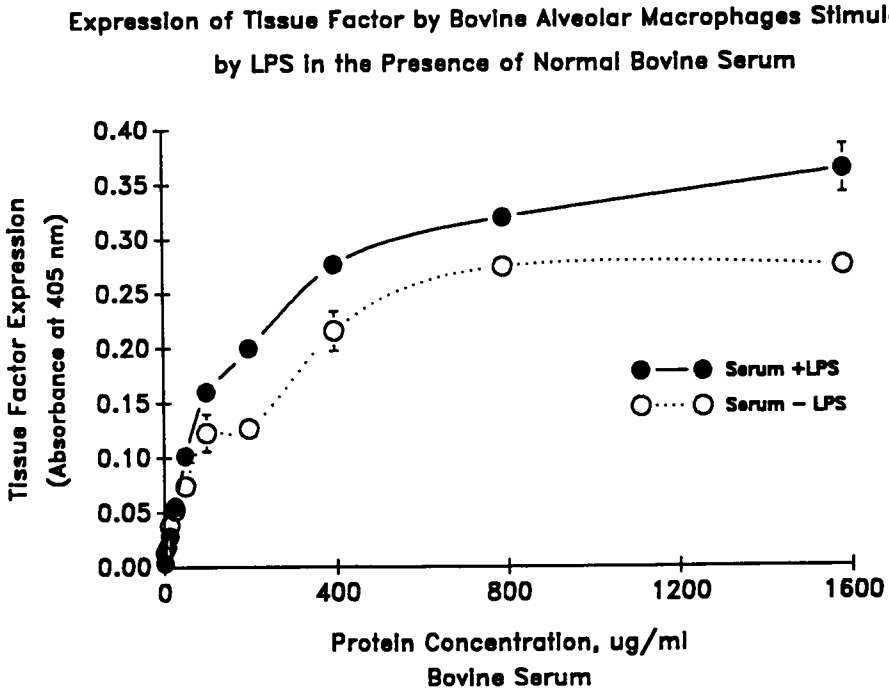


FIG. 4. Effects of various protein pools, obtained by fractionation of normal bovine serum using Bio-Rex 70 column, on LPS-induced tissue factor expression by bovine alveolar macrophages. Macrophages were exposed to +/- 1 ng/ml LPS from *E. coli* 055:B5. Values are the means +/- standard deviation (S. D.) of quadruplicate samples of a representative experiment.

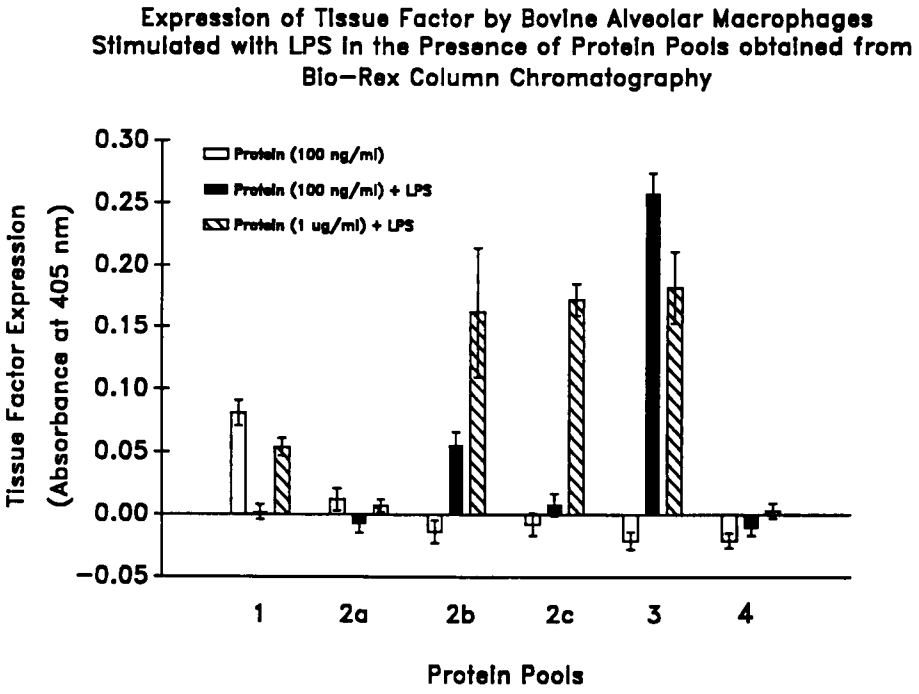


FIG. 5. Effect of dose response of "pool 2c" on LPS-induced tissue factor expression by bovine alveolar macrophages. Macrophages were exposed to +/- 1 ng/ml of LPS from *E. coli* 055:B5. Values are means +/- S. D. of quadruplicate samples of a representative experiment. This assay was done three times. Where error bars are not visible, the S. D. was smaller than the size of the symbol used on the graph.

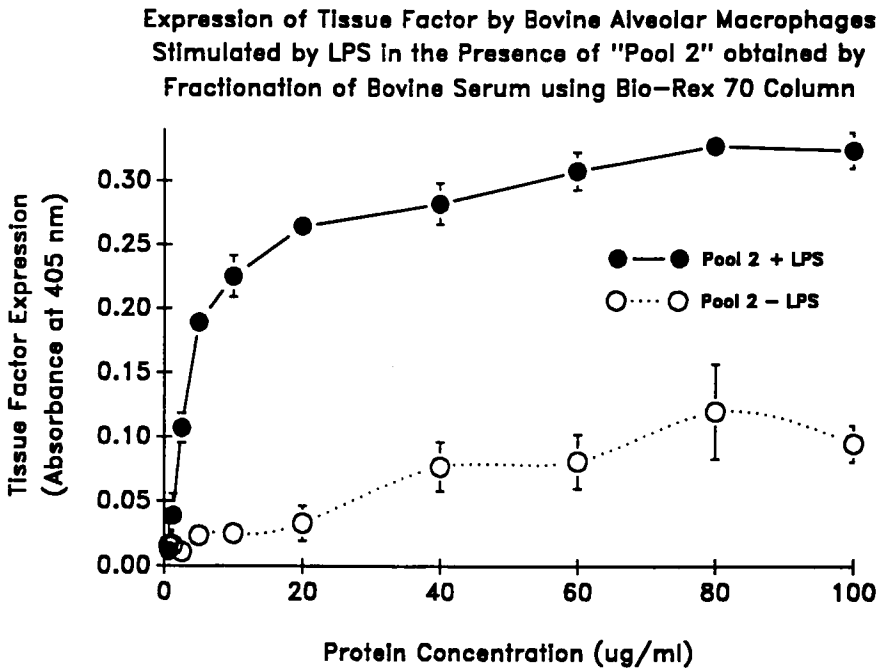


FIG. 6. Effect of various concentrations of HPLC purified (fraction 70 + 71 of run 1) "pool 2" on LPS-induced tissue factor expression by bovine alveolar macrophages. LPS from *E. coli* was used at 1 ng/ml. Values are the means +/- S. D. of quadruplicate samples of a representative experiment. Where error bars are not visible, S. D. was smaller than the size of the symbol used on the graph.

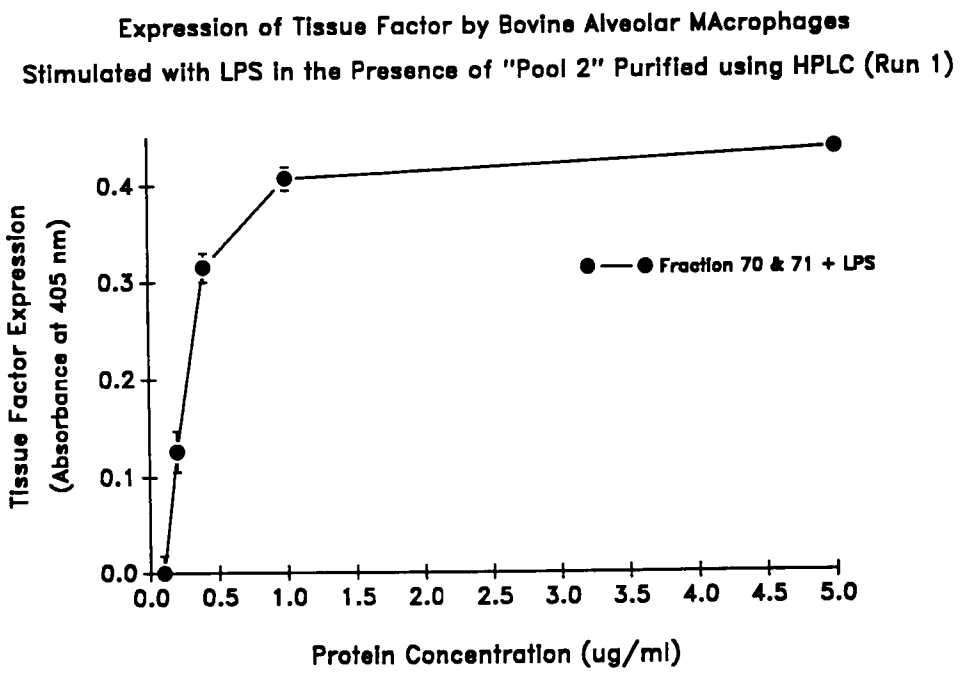


FIG. 7. Effects of fractions obtained by run 1 and 2 of HPLC purification of "pool 2" on LPS-induced tissue factor expression by bovine alveolar macrophages. LPS from *E. coli* 055:B5 was used at 1 ng/ml. Values are the means +/- S. D. of quadruplicate samples of a representative experiment.

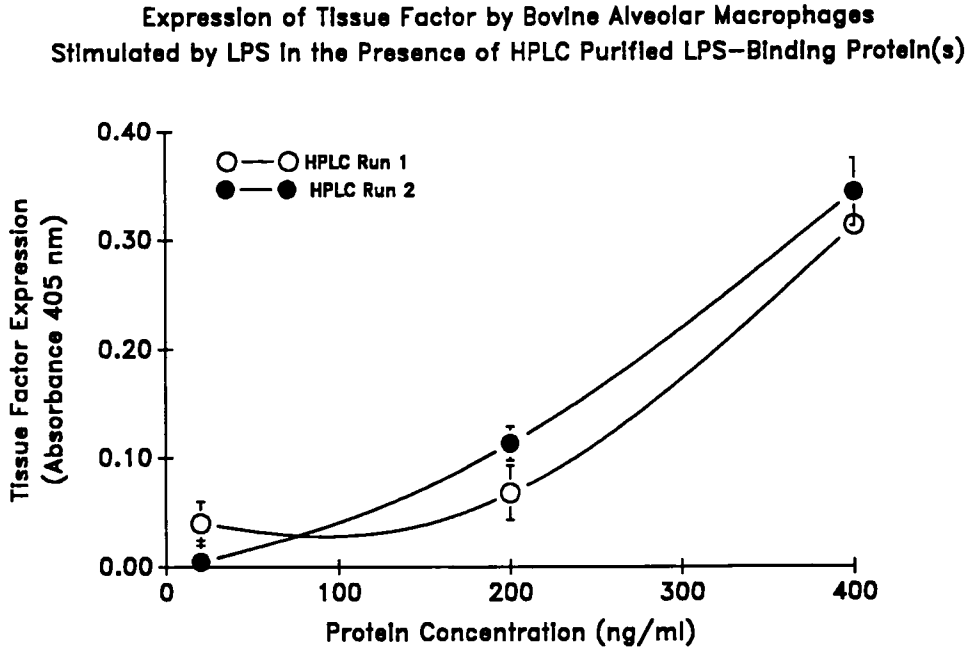


FIG. 8. Effects of dose response of pools 5 and 6 collected from HPLC purification of "pool 2c" on LPS-induced tissue factor expression. LPS from *E. coli* 055:B5 was used at 1 ng/ml. Values are the means +/- S. D. of quadruplicate samples of a representative experiment.

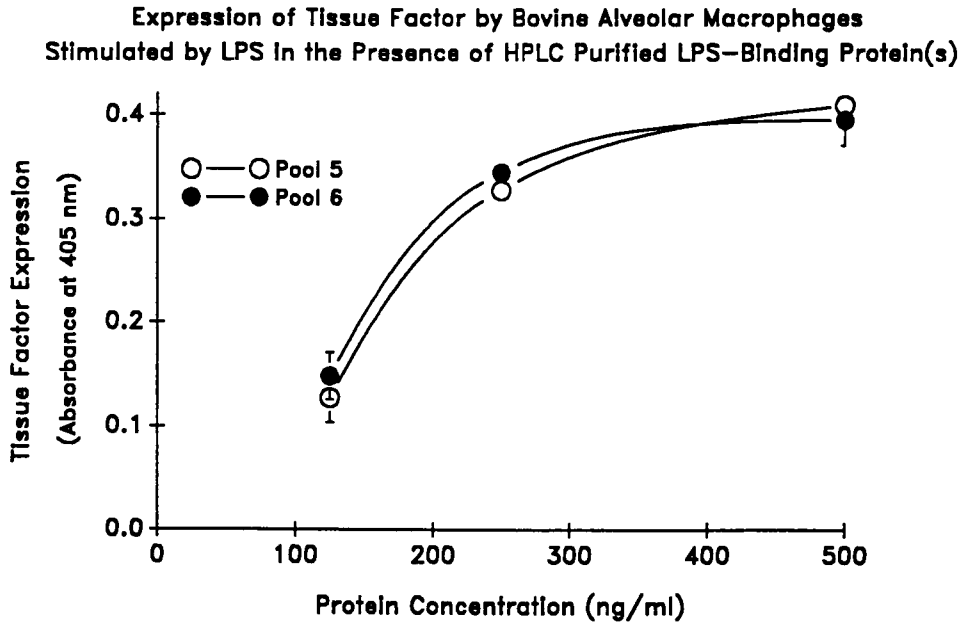


FIG. 9. Effects of dose response of fractions (22.13 and 38.55 minute pools) collected by HPLC purification on LPS-induced tissue factor expression by bovine alveolar macrophages. Macrophages were stimulated with/without 1 ng/ml LPS from *E. coli* 055:B5. Values are the means +/- S. D. of quadruplicate samples of an experiment.

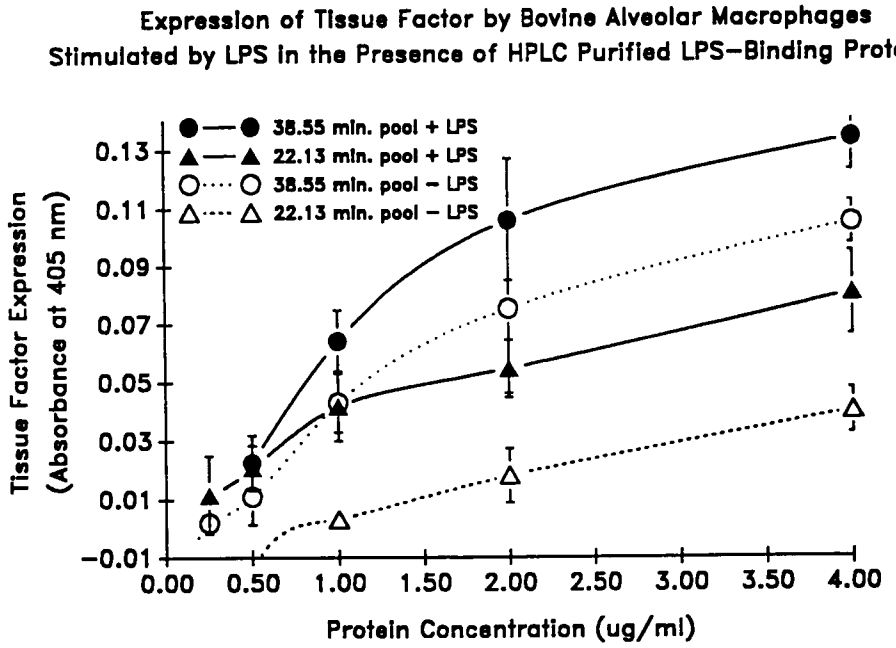


FIG. 10. Effects of dose response of NBS, "pool 2c" and fraction 70 + 71 (HPLC, run 1) on LPS-induced tissue factor expression by bovine alveolar marcophages. Values are the means +/- S. D. of quadruplicate samples of representative experiments. Where error bars are not visible, the S. D. or S. E. was smaller than the size of the symbol used on the graph.

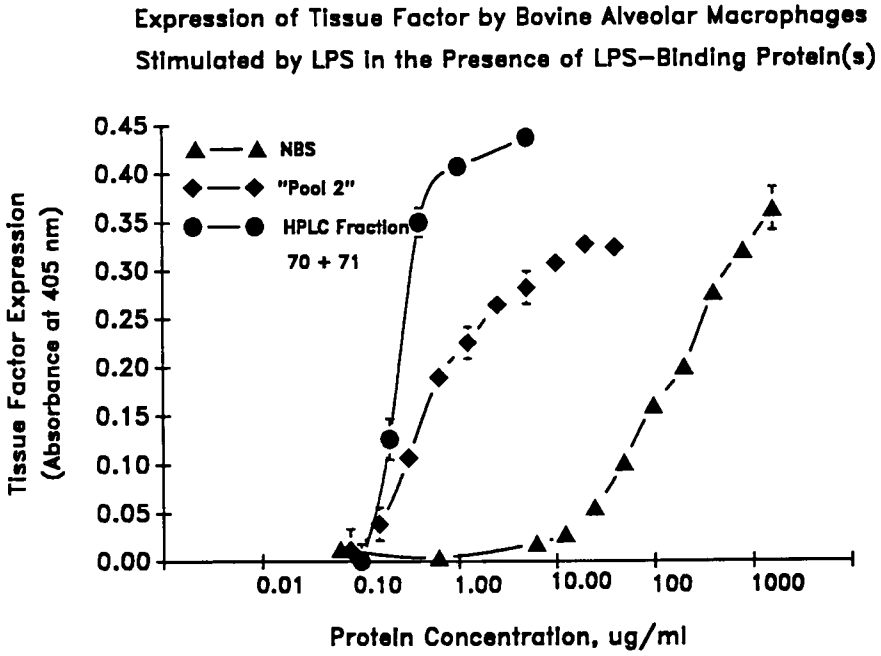


FIG. 11. Effect of heating on protein-related tissue factor expression by LPS-stimulated bovine alveolar macrophages. "Pool 2, " HPLC purified fractions obtained at 22.13 and 38.55 minutes were heated to 72℃ for 1 hour prior to treating macrophages. The protein concentrations used were; "Pool 2" (20 μg/ml), HPLC purified proteins (2 μg/ml). LPS from *E. coli* 055:B5 was used at 1 ng/ml. Values are the means +/- S. D. of quadrupliate samples of an experiment.

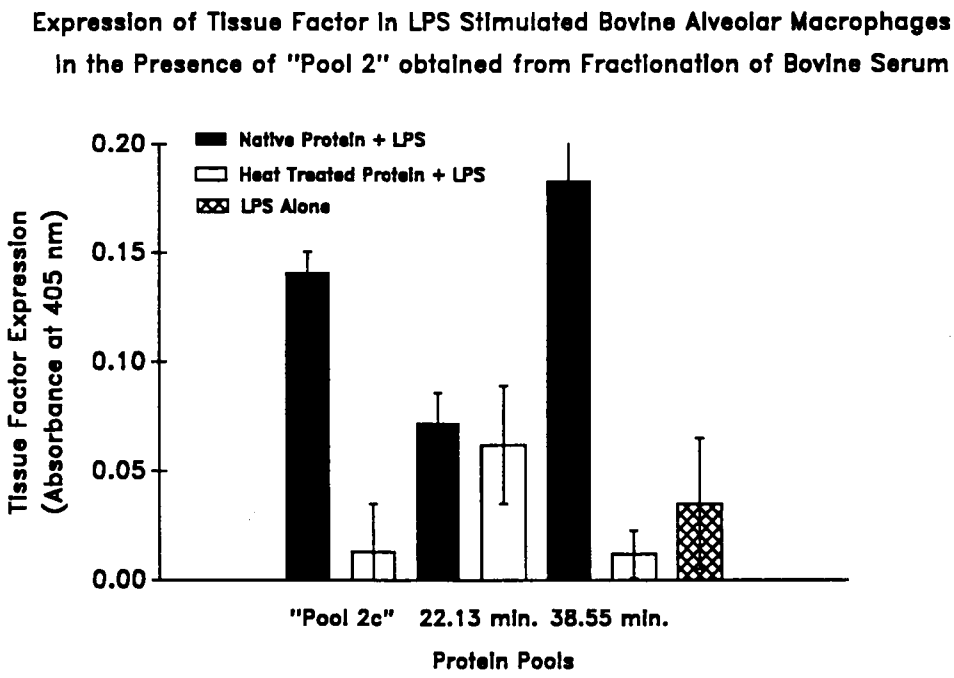


FIG. 12. Effect of polymyxin B on LPS-induced tissue factor expression by bovine alveolar macrophages. *E. coli* 055:B5 LPS was incubated with 10 μg/ml of polymyxin B for 40 minutes prior to treating macrophages with LPS and protein. LPS was used at 1 ng/ml. Protein concentrations used were: "Pool 2" (20 μg/ml), HPLC purified proteins (2 μg/ml). Values are the means +/- S. D. of quadruplicate samples of a representative experiment. This experiment was done twice.

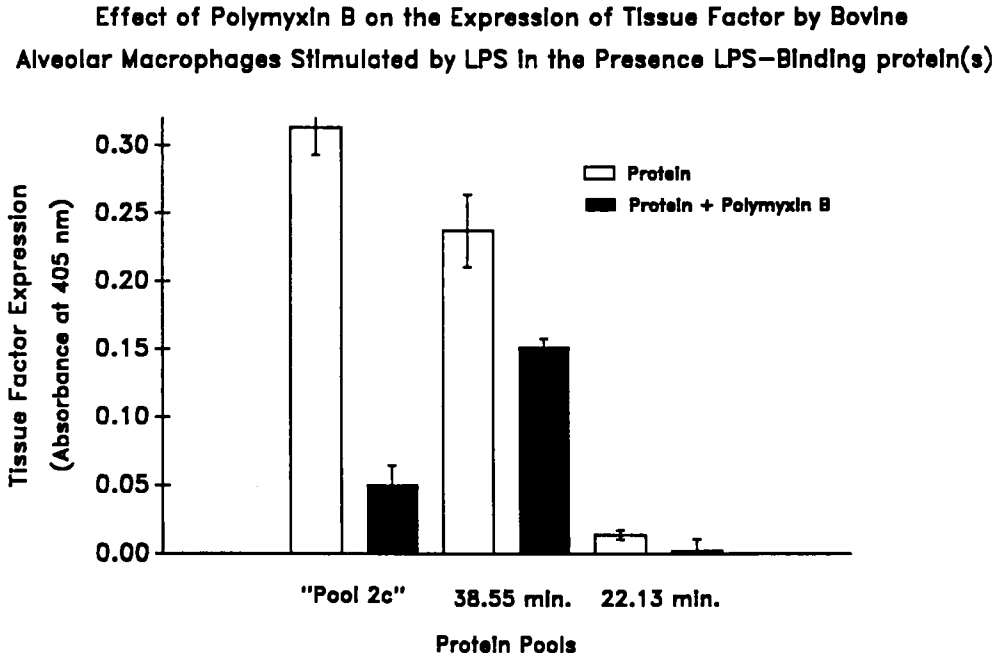


FIG. 13. Effect of anti-CD14 monoclonal antibody on tissue factor expression by LPS-stimulated bovine alveolar macrophages. The effect of the monoclonal antibody 60bd is shown (10 μg/ml), and isotype-specific control murine IgG treatments is seen in the same graph. Macrophages were exposed to "pool 2c" (20 μg/ml) and HPLC purified proteins eluted at 22.13 and 38.55 minutes (2 μg/ml) +/- antibodies. Values shown are the means of quadruplicate samples, n = 4.

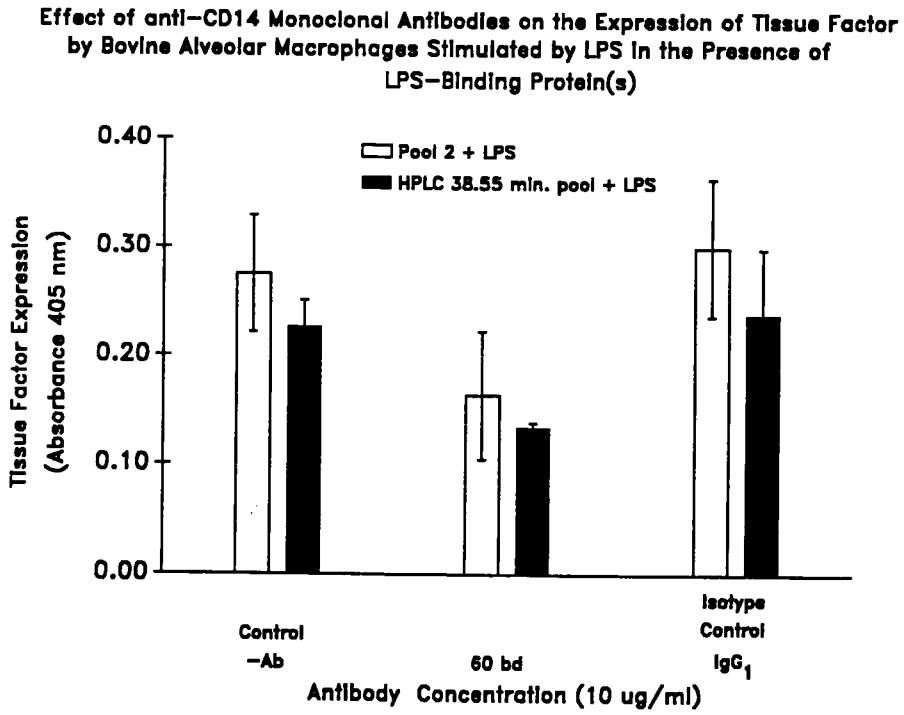
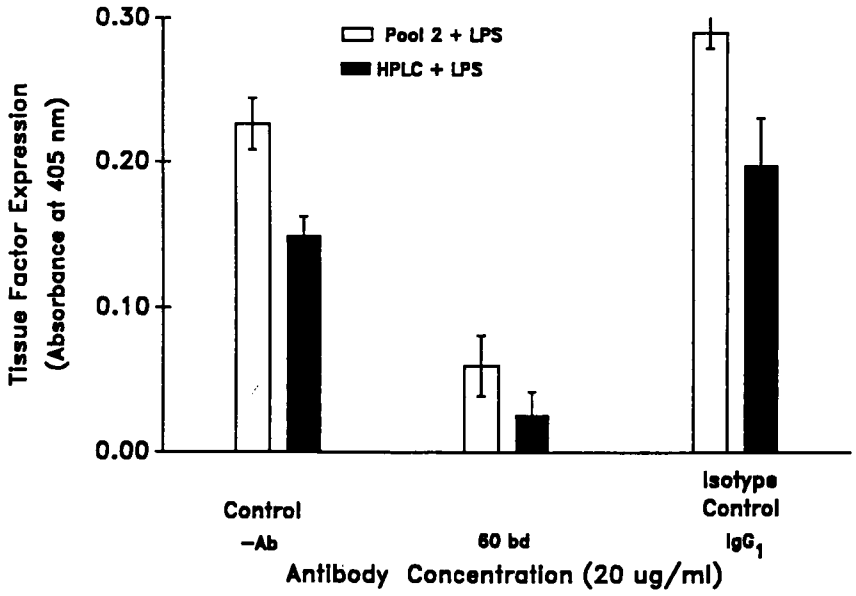


FIG. 14. Effect of anti-CD14 monoclonal antibody on tissue factor expression by LPS-stimulated bovine alveolar macrophages. The effect of the monoclonal antibody 60bd is shown (20 μg/ml), and isotype-specific control murine IgG treatments is seen in the same graph. Macrophages were exposed to "pool 2c" (20 μg/ml) and HPLC purified proteins eluted at 22.13 and 38.55 minutes (2 μg/ml) +/- antibodies. Values shown are the means +/- S. D. of quadruplicate samples. This assay was done twice.



PART 5

SUMMARY

SUMMARY

Endogenous regulatory mechanisms have been developed by the host in response to bacterial infections. As seen from preceding chapters it is not endotoxin itself that causes the ultimate harm to the host, rather, it is the overwhelming response by the immune system.

Some of the proteins involved in the regulation of LPS exposure do so by either potentiating or attenuating effects of LPS. Recent studies have identified from the rabbit, murine and human serum a protein called LBP that upon binding to LPS with high affinity augments LPS effects. LPS-LBP complex has been shown to stimulate cells such as monocytes and macrophages through a membrane bound receptor called CD14.

Cattle, just any other species have been shown to be sensitive to LPS infusion. Endotoxemia has been shown to result in large losses in the cattle industry. However, to date, published literature does not address the presence of any bovine serum component(s) that make cattle very sensitive to endotoxin.

In the present dissertation project, a 60 kDa LPS binding protein has been identified. This protein has been shown to have considerable homology to the previously characterized LBPs. Comparison of the first 20 amino acids of the 60 kDa bovine serum LPS-binding protein NH_2-terminal has shown that it is significantly homologous not only to LBPs, but also to bactericidal/permeability-increasing protein. Furthermore, adult normal bovine serum and protein fractions

containing the bovine serum protein of interest has been shown to shift the threshold of bovine alveolar macrophages in the presence of nanogram/ml concentrations of LPS. The stimulatory effect of LPS-LBP complex has been shown, using anti-human CD14 monoclonal antibodies, to function through the CD14 receptor, just like in other species. Results from tissue factor assays done in the presence of heat denatured protein fractions and polymyxin B, an inhibitor of LPS-binding has shown that the 60 kDa LPS-binding protein isolated from bovine serum is heat labile and binds to the lipid A region of LPS.

In conclusion, as seen from preceding chapters, it seems appropriate to end this dissertation by mentioning Lewis Thomas's view that "disease usually results from the inconclusive negotiations for symbiosis, an overstepping of the line by one side or the other, a biologic misinterpretation of borders."

VITA

Lajwanti Sobhrajmal Khemlani was born in April 1960 in Poona, in the state of Maharashtra, India. She was brought up and received her original education in that city.

Her secondary education was at Rosary High School and her higher secondary education at Saint Annes School and Nowrosjee Wadia college. She did her undergraduate studies at Wadia College and Ferguson college receiving a Bachelors degree in Chemistry from Poona University in 1982.

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