ROLE OF LIPOPOLYSACCHARIDE ON *PASTEURELLA*

HAEMOLYTICA A1 LEUKOTOXIN STRUCTURE

AND FUNCTION

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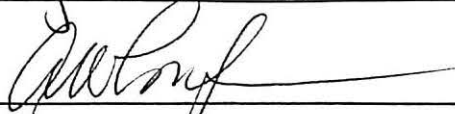the Degree of
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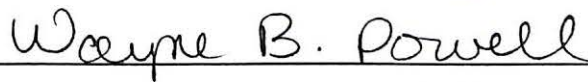
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LKT: *Pasteurella haemolytica* A1 leukotoxin

LPS: Lipopolysaccharide

CCS: Concerntrated culture supernatant

TNF: Tumor necrosis factor

PCR: Polymerase chain reaction

RT-qcPCR: Reverse transcriptional quantitative competitive PCR

MAb: Monoclonal antibody

CD: Cluster of differential molecule

RTX: Repeat-in-toxin

LTB4: Leukotriene B4

KDO: 2-keto-3-deoxy-octosonic acid

SDS: Sodium dodecyl sulfate

PAGE: Polyacrylamide gel electrophoresis

LDH: Lactate dehydrogenase

BL: Bovine lymphoma

DEPC: Diethyl phosphoryl cyanide

Chapter I: Introduction

Cattle shipping fever is an economically important disease. In the United States, its associated economic loss costs the beef and dairy industries about $800 million annually (Drummond et al, 1981). The lesions of cattle shipping fever are characterized as an acute fibrinous brochopneumonia with toxemia (Lopez, 1995). Grossly, the pneumonic pasteurellosis lesions are localized on the cranioventral regions of the lung and consist of severe congestion, hemorrhage, massive exudation of fibrin and red discoloration (Lopez, 1995). Microscopically, fibrin and inflammatory cells exudation into the air space and fibrin thrombosis in lymphatic vessels are observed along with areas of coagulative necrosis. The intensive neutrophil infiltration of the lung is most prominent (Lopez, 1995).

Several factors are frequently associated with initiation of this disease, such as stress, viral and/or bacterial infection. One of the major etiologic factors is a gram-negative bacterium e.g. *Pateurella haemolytica* biotype A serotype 1. *Pasteurella haemolytica* A2 is the dominant type in the nasopharynx of healthy cattle, and *P. haemolytica* A1 is barely detectable (Frank and Smith, 1983). However, after viral infection and/or stress, the amount of fibronectin on the cell surface decreases, and/or the function of ciliated cells may be altered. These factors may favor bacterial adhesion and colonization (reviewed by Whiteley et al, 1992), so that *P. haemolytica* A1 proliferates rapidly, and subsequently the bacteria are then transferred to the lung by inhalation droplet. *P. haemolytica* A1 is the predominant type isolated from the pneumonic lung of calves with shipping fever (Frank and Smith, 1983). The proliferation of bacteria in the

lung results in activation of alveolar macrophages, followed by cytokine release and activation of the pro-inflammatory cascade, finally pneumonic pasteurellosis occurs (Weekley 1998b). The pathogenesis of cattle shipping fever in detail is still under investigation.

P. haemolytica A1 produces several virulence factors such as leukotoxin (LKT), fimbriae, lipopolysaccharide (LPS), capsular polysaccharide (Confer et al, 1990), protease, sialidase, and outer-membrane components (Weekley, 1998b).

LKT is one of the major virulence factors in the pathogenesis. Inoculation of LKT-deficient P. haemolytica A1 in the cattle lung induced less extensive lesions than those produced by wild-type strain (Froshauer et al, 1993). Cattle with high-titer anti-LKT antibodies were more resistant to shipping fever (Conlon et al, 1991). LKT is secreted from P. haemolytica into medium at the logarithmic growth phase. It acts specifically on ruminant leukocytes (Confer et al, 1990) and platelets (Clinkenbeard and Upton, 1991). As Confer and his colleagues (1995) reviewed, at low dose, LKT stimulates leukocytes. After stimulation, neutrophils produce arachidonic acid metabolites, release secondary granules, promote oxidative burst, and induce cell apoptosis (Srevens and Czuprynski, 1996). After stimulation, mononuclear phagocytes release IL-1 and TNF-α cytokines (Yoo et al, 1995) and mast cells release histamine. LKT also inhibits mitogen-mediated blastogenesis of bovine lymphocytes in vitro. At high concentration level, LKT lyses leukocytes and platelets. Neutrophils and macrophages play important roles in the protection response, therefore, lysis of these cells decreases the immune ability. The mediators induced by LKT can "increase vascular permeability, stimulate chemotaxis of neutrophils, promote leukocyte adhesion

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to endothelial cells, stimulate synthesis of platelet activating factor and increase pro-coagulant properties of endothelium" (Confer et al, 1995). Therefore, LKT plays an important role in the pathogenesis of cattle shipping fever.

LPS is another major virulence factor in the pathogenesis of cattle shipping fever. Deposition of *P. haemolytica* A1 LPS into the sheep's lungs resulted in a marked influx of neutrophils into the lung and stimulated the development of lesions of pnuemonic pasteurellosis (Brogden et al, 1984). LPS, e.g. endotoxin, is a common virulence factor of all gram-negative bacteria. LPS acts on myeloid cells and endothelial cells through molecular interaction. For example, LPS interacts with LPS binding protein (LBP) (Kirland et al, 1993) and soluble CD14 molecules (Maliszewski et al, 1985) in the serum. LPS also interacts with mCD14 (Wright et al, 1990), Toll-like receptor (Yang et al, 1998), β2 integrin (Wright and Jong, 1986) on the myeloid cells. LPS-sCD14 complex acts on endothelial cells directly (Haziot et al, 1993). Through these interactions, cytokines are induced and released into serum, which further activate myeloid cells and endothelial cells. Thus, LPS stimulates neutrophil adherence, alters leukocyte function and damages endothelial cells (Confer et al, 1990), which lead to vascular permeability, leukocytic adherence to endothelial cells and increased intravascular coagulation. Hence, LPS from *P. haemolytica* also plays an important role in the pathogenesis of the cattle shipping fever.

LPS and LKT may affect each other. Pre-treating the rabbit lung with LPS enhanced thromboxane release and other action in response to *E. coli* hemolysin (Walmrath et al, 1994). In addition, genetic mutations on the LPS synthesis genes reduce *E. coli* hemolysin (another Repeat-in-Toxin toxin family member) production and

activity (Bauer and Welch, 1997. Stanley et al, 1993). Some researchers proposed that LPS may be necessary for LKT function (Czuprynski and Welch, 1995).

A better understanding of the interactions among LPS, LKT and host cells may be helpful in control and treatment of cattle shipping fever. In the following chapter, the literature on LPS and LKT will be reviewed, followed by presentation of a summary, problem statement and experiment research on the relations among LPS, LKT and target cells.

Chapter II: Literature Review I

Genetics, Structure and Function of

Pasteurella haemolytica leukotoxin

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LKT is a member of the RTX toxin family. Sequence alignments indicate that LKT, *Escherichia coli* hemolysin (HlyA), *Actinobacillus actinomycetemcomitans* leukotoxin, *A. pleuropneumoniae* hemolysin are highly homologous to one another. According to their target cells specificity, RTX toxins are divided into two classes, leukotoxins and hemolysins. Leukotoxins act on leukocytes only. Hemolysins lyse erythrocytes as well as various nucleated cell types.

1. Genetic organization of *Pasteurella haemolytica* leukotoxin

The reported RTX toxins have the same genetic organization with the exception of *A. pleuropneumoniae* hemolysin (Chang et al, 1991). The leukotoxin related genes (lkt), gene C, A, B and D, form a gene cluster on the bacterial genome (Fig. 1.1). From upstream to downstream, the genes are lktC, lktA, lktB, lktD, which encode 19.9, 102, 79.6 and 54.7 kD proteins (Highlander et al, 1989). The regions between open-reading-frame (ORF) are 15bp, 75bp and 11bp respectively (Highlander et al, 1989). The CABD genes share a common promoter (Highlander et al, 1989). Analysis of lktCABD mRNA sequence indicates that there is a stem-loop transcriptional terminator between gene A and B (Highlander et al, 1990). Via antitermination, the transcript of lktCA may continue into the downstream lktB and lktD genes (Highlander et al, 1990). Hence, two different length mRNA, lktCABD and lktCA, are transcribed.

RTX gene expression is tightly regulated. At 130 bp upstream from the transcription start site of lkt, three upstream transcriptional activation sites are found. The three elements can bind DNA binding protein and integration host factor (IHF). The

bound proteins then interact with RNA polymerase on the promoter region by DNA bending, and activate LKT expression (Highlander and Weinstock, 1994). The promoter of *E. coli* hemolysin is negatively-regulated by Hha and positively-regulated by RfaH. Godessart and his coworkers (1988) reported that production of *E. coli* hemolysin (HlyA) was increased in hha mutant. Later, Jubete and co-worker (1995) demonstrated that Hha protein interacted on a 200-bp region within hlyC gene (designated hlyM), and silenced the expression of hlyCABD. Surprisingly, several LPS synthesis related genes have some functions on RTX toxin expression. For example, genes rfaH and galU are required for LPS biosynthesis. Mutations in them strongly reduce HlyA production (Wandersman and Letoffe, 1993). By primer extension, RfaH is demonstrated to act as an anti-terminator and enhances the elongation of *E. coli* hlyCABD mRNA transcription (Leeds and Welch, 1996). RfaC, an LPS biosynthesis gene may also have pleotropic effects on HlyA. Mutations in rfaC gene decreased both the expression and activity of HlyA (Bauer and Welch, 1997).

LktA is a structural gene, which encodes leukotoxin. LKT is a pore-forming protein. Post-translational modification of LKT is necessary for LKT activity, following which, the active LKT is secreted into culture medium. The structure and function of LKT will be discussed later.

LktC encodes an acyl transferase, which acylates and activates LKT. LktC is similar to HlyC, and they share a sequence similarity of 66% (Strathdee and Lo, 1987. Forestier and Welch, 1990). Nicaud et al (1985) found that hlyC gene product can activate HlyA. Later, Issartel and co-workers reported (1991) that HlyC transferred acyl from acyl carrier protein (ACP) to HlyA thereby activating HlyA. Acyl-CoA could not be

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used as donor for acyl to replace acyl-ACP. Stanley and his colleagues (1994) demonstrated that acylation of two internal lysine residues was required for HlyA activity. The fatty acid was amide linked to the amino acid residues (Stanley et al, 1994). The fatty acids include myristic, palmitic, palmitoleic and cis-vaccenic acids (Hughes et al, 1992). LktC has the similar function to that of HlyC. Fedorova and Highlander (1997) inserted a CAT cassette into lktC gene and inactivated lktC but did not affect the expression and secretion of pro-LKT. Inactivated LKT was obtained in the culture medium. Therefore, lktC gene product is also necessary to activate LKT. The acyl-modified amino acids on LKT are Lys-557 and Asn-684 residues (reviewed by Stanley et al, 1998). Similar to HlyA, the different fatty-acyl groups linked to LKT do not affect LKT target cell specificity, although the overall LKT activity is sightly different (Hormozi et al, 1998). Although HlyC and LktC share high homoloy and similar function, lktC gene can not complement hlyC gene. Forestier and Welch (1990) observed that HlyC was able to activate HlyA and LKT, but LktC could not activate HlyA. Hence, Forestier and Welch (1990) proposed that mechanism of activation by LktC and HlyC are similar, but the structural requirements for interaction are different. The active form of HlyC is homodimer (Hardie et al, 1991). It is not known yet whether the active form of LktC is a homodimer.

The acylation of LKT is required for LKT activity, but acylation is not required for LKT export (Fedorova and Highlander, 1997). After acylation in cytoplasma, LKT is secreted across inner and outer membrane into culture medium through a type-I export system which requires uncleaved C-terminal recognition sequence (Nicaud et al, 1986). HlyB, hlyD and tolC encode the HlyA secretion apparatus. HlyB encodes an inner

membrane protein with a cytoplasmic ATP-binding cassette, which couple ATP binding, hydrolysis and protein conformational change. The free energy associated with ATP hydrolysis is used for HlyA export (Koronakis and Hughes, 1993). HlyD encodes an inner membrane protein, which has a single transmembrane segment, a small N-terminal cytoplasmic portion and a large C-terminal periplasmic portion (Schulein et al, 1992). Therefore, HlyD is proposed to bridge the inner membrane and outer membrane for LKT export. TolC is a minor outer membrane protein, encoded by a gene out of hly locus (Wandersman and Delepelaire, 1990). Electron microscopy reveals that TolC forms an trimeric pore-like complex with C-terminal *domain* in the periplasm (Koronakis et al, 1997). LktB and HlyB share a sequence similarity of 90.5%, and LktD and HlyD share a sequence similarity of 75.6% (Forestier and welch, 1990). Therefore, it is proposed that LktB and LktD have the similar corresponding function to HlyB and HlyD respectively. The tolC gene in *Pasteurella haemolytica* has not been reported.
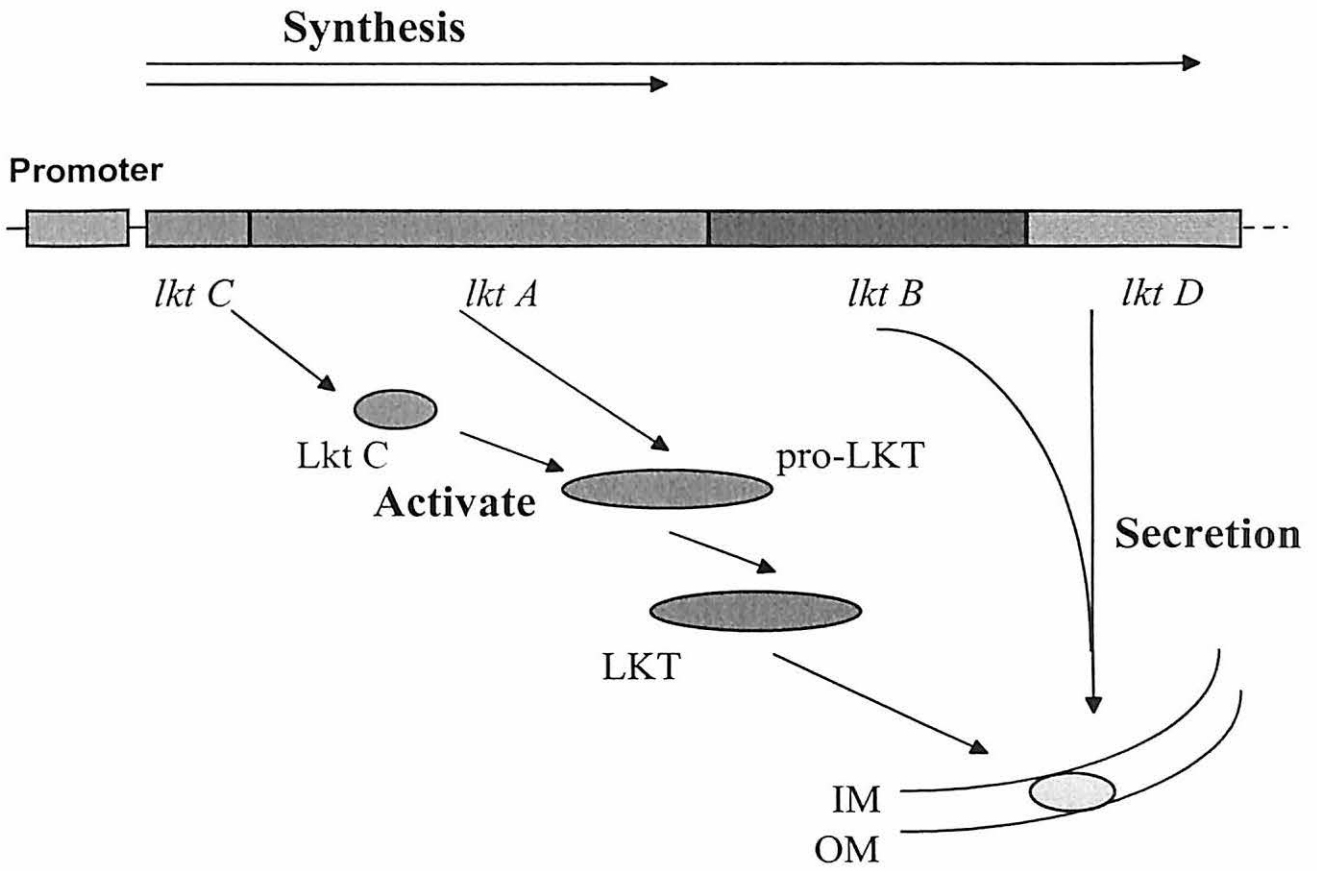
Fig. 1.1 Leukotoxin synthesis, maturation, and exported by *Pasteurella haemolytica* (According to Highlander et al, 1989 and Stanley et al, 1998).

2. Structure of *Pasteurella haemolytica* leukotoxin

LKT is a heat labile, water soluble protein with molecular weight of 105 kDa on the analytic SDS-PAGE (Chang et al, 1987). According to the LKT amino acid sequence, the molecular weight of LKT is about 102kDa (Highlander et al, 1989). However, the native LKT molecular weight is about 160-8000kDa as determined by gel filtration chromatography (Moiser et al, 1986. Chang et al, 1987. Clinkenbeard et al, 1995). This high appearing molecular weight indicates that LKT tends to form aggregate (Clinkenbeard et al, 1995).

LKT is a single chain polypeptide with 953 amino acid residues without disulfide bonds (Stanley et al, 1998). Like other RTX toxins, LKT has tandemly arranged repeats of a nine-amino-acid sequence (L/I/F-X-G-G-X-G-N/D-D-X).

The cell-binding domain is separated from lytic domain. Cruz et al (1990) found that an LKT mutant without segments of amino-proximal hydrophic region could agglutinate bovine lymphoma, BL3 cell. This mutant protected BL3 cells from lysis by the wild-type LKT in a competitive way. Bauer and Welch (1996) confirmed that binding and lysis by HlyA were separated events.

Furthermore, Forestier and Welch (1991) proposed that LKT has five domains, each of which has different function. They used LKT-hlyA hybrids to find that hybrids with amino-terminal 169 residues of LKT lysed BL3 cells more efficiently than Raji cells. Therefore, they suggested that the N-terminal domain of LKT is related to ruminant target cell specificity. Opposite to this hypothesis, McWhinney et al (1992) reported that the leukocytic potential of LKT maps to the C-terminal half of the protein. The

leukocytic potential domain of *A. pleuropneumoniae* hemolysin is separated from the erythrocytic potential domain.

The domain from residues 89 to 392 of LKT forms several hydrophobic segments which is proposed to form the transmembrane pore. The hydrophobicity of this domain may make LKT tend to aggregate and lose activity. As mentioned above, the molecular weight of aggregate LKT can reach 8000kDa, and chaotropic agents cause disaggregation and enhance activity of LKT (Clinkenbeard et al, 1995).

Following this domain, there is about 300 amino acids rich in beta-turn. At this region, two lipids are linked to residues lys-554 and asn-684 (Stanley et al, 1998). The function of this domain has not been reported.

The next domain contains Gly-Asp repeats, which can bind calcium. Boehm and co-workers (1990) reported that extracellular calcium binding was required for *E. coli* hemolysin binding to erythrocytes and nucleated cells. Cruz et al (1990) also reported that calcium and LktC activation is required for LKT binding to cultured BL3 cells. However, Clinkenbeard and co-workers (1989) demonstrated that extracellular calcium was not necessary for LKT binding to bovine lymphoma cells, but calcium is required for LKT to lyse the target cells. This domain is also necessary for *A. actinomycetemcomitans* leukotoxin to recognize human target cells (Lally et al, 1994).

The C-terminal 70 amino acid domain is necessary for LKT secretion from bacteria into medium. It can be recognized by LktB/D transporter and secreted out of the bacteria. Nicaud and his colleagues (1986) constructed an expression plasmid which carried the 3'-end of hlyA gene, and transformed the plasmid into *E. coli* strain with hemolysin export apparatus hlyB/D. The C-terminal 23kD peptide was efficiently

secreted. Although the C-terminal sequence of LKT and HlyA don't have significant similarity in primary sequence, they share a similar structure motif of helix-turn-helix followed by by strand-loop-strand (Zhang et al, 1993). Replacing the C-terminal 50 amino acids of HlyA with C-terminal 50 amino acids of LKT does not affect the secretion of hybrid protein (Zhang et al. 1993).

3. Function of *Pasteurella haemolytica* leukotoxin

LKT plays an important role in the pathogenesis of cattle shipping fever. It specifically acts on ruminant neutrophils (Kaehler et al, 1980) and platelets (Clinkenbeard and Upton, 1991). Confer and his coworkers reported that LKT acted on ruminant neutrophils from cattle, sheep, goats, deer, Saiga antelope, Grant's gazelle and Sable antelope (Confer et al, 1990). Neutrophils from non-ruminant species are resistant to LKT (Confer et al, 1990).

The first step for LKT action is binding to target cells. It is generally believed that LKT binding specificity determines LKT target cell specificity. Brown and co-workers (1997) demonstrated by flow cytometry that LKT binds to bovine leukocytes but not pig and human leukocytes. However, others have found that LKT could bind to human lymphocytes Raji cells (Sun et al, 1997) and that *E. coli* hemolysin could bind to artificial protein-free lipid vesicle (Ostolaza and Goni, 1995). LKT does not bind to protease K treated bovine neutrophils, which may indicate that some proteins on the cell surface are involved in the binding process (Brown et al, 1997). Recently, Lally and his colleagues (1997) found that anti-β2 integrin antibody inhibited HlyA activity and transfected nonsusceptible cells with β2 integrin became susceptible to HlyA and *A. actinomycetemcomitans* leukotoxin. Therefore, they proposed that β2 integrin is a receptor for *A. actinomycetemcomitans* leukotoxin and HlyA.

After binding to target cells, RTX toxins change their conformation (Moayeri and Welch, 1997), and insert into target cell membranes to form transmembrane pores (Bhakdi et al, 1986. Murphy et al, 1995). Insertion is a different event from binding. Some LKT mutants without transmembrane pore-forming domain still bind BL3 cells

well and are competitors of wild-type LKT (Cruz et al, 1990). The initial pore size caused by LKT is sightly less than 0.9nm (Clinkenbeard et al, 1989). LKT causes rapid leakage of intracellular K^+ and subsequent cell swelling (Clinkenbeard et al, 1989). Hypertonic sucrose solution prevents cell swelling, but does not interfere with leakage of intracellular potassium. The above events are not dependent on the presence of extracellular calcium (Clinkenbeard et al, 1989).

LKT may induce extracellular calcium influx through a voltage-gated channel (Hsuan et al, 1998). The intracellular calcium concentration can reach 1.5μM from 0.1μM of resting intracellular concentration. The requirement for extracellular calcium for LKT function differentiates the LKT signal pathway from the LPS signal pathway due to the fact that LPS can induce intracellular calcium concentration increase in the absence of extracellular calcium (Hsuan et al, 1998). The increased intracellular calcium levels induced by LKT may signal the following events (Cudd et al, 1999): 1) activation of phospholipase, 2) release of cytokines, 3) apoptosis, 4) degranulation and production of reactive oxygen intermediates, and 5) lysis.

Clarke and his colleagues (1994) reported that *P. heamolytica* A1 increased the production of leukotriene B4 (LTB4) by tissue chamber model. Later these workers demonstrated that LKT induced LTB4 production from bovine neutrophils *in vitro* (Clinkenbeard et al, 1994). Recently, Wang and his co-workers (1998) found that LKT activates phospholipase A2 (PLA2), which then cleaves eicosanoids from membrane phospholipids in bovine lymphoma cells. The eicosanoids are used to synthesize LTB4 by 5-lipoxygenase. The liberation of arachidonic acid from phospholipids is the rate-

limiting step for LTB4 production. The activation of PLA2 may be mediated by LKT activation of phospholipase D (Bauldry and Wooten, 1997. Wang et al, 1999).

LKT induces cytokine synthesis and release. Yoo and his colleagues (1995) found that purified LKT induced expression of IL-1 and TNF-α as detected by Northern blot. They also demonstrated that 0.5 U/ml LKT started to induce bovine alveolar macrophages to transcribe TNF-α and IL-1β mRNA and reach maximal expression at 1 U/ml LKT. At higher concentration of LKT, bovine alveolar macrophages were lysed. On the other hand, Stevens and Czuprynski (1995) proved that monokine release from bovine monocytes induced by the partially purified LKT was due to the contaminated LPS in the samples. They found that heat-treated LKT or LKT treated with neutralizing monoclonal antibodies lost its activity in inducing cytolysis. However, it still induced monokine release. Recently, Hsuan and his colleague (1999) reported that LKT induced NF-κB activation and intracellular calcium elevation in LKT-susceptible bovine alveolar macrophage (BAM), and subsequently induced expression of IL-1β, TNF-α and IL-8. LPS induced expression of IL-1β, TNF-α and IL-8 in all cell types (Hsuan et al, 1999). If pretreated BAM cells with BAPTA/AM to chelate calcium, no expression of IL-1β, TNF-α and IL-8 was detected after treatment with either LKT or LPS (Hsuan et al, 1999). Tyrosine kinase inhibitor blocked LKT-induced expression of IL-1β, TNF-α and IL-8 in BAM and blocked LPS-induced expression of IL-1β (Hsan et al, 1999).

Sub-lytic doses of LKT induce bovine neutrophil apoptosis. After incubation with sub-lytic dose of LKT, bovine leukocytes demonstrate morphologic changes such as cytoplasmic membrane blebbing, chromatin condensation and margination, which is consistent with apoptosis (Stevens and Czuprynski, 1996). Later, two research groups

reported that LKT induced bovine lymphoma cells to undergo internucleosomal DNA fragmentation which is a hallmark of cell apoptosis (Wang et al, 1998b. Sun et al, 1999).

Sub-lytic doses of LKT induce degranulation and generation of oxygen-derived free radicals. Maheswaran and co-workers (1992) reported that LKT-containing *P. haemolytica* culture supernatant induced bovine neutrophil respiratory burst to cause release of free oxygen radicals and degranulation. Leukotoxin-neutralizing monoclonal or polyclonal antibodies abrogated degranulation and respiratory burst, anti-LPS antibodies did not. High dose LKT also suppresses respiratory burst due to cytolysis of bovine neutrophils. Therefore, they proposed that LKT induced neutrophil degranulation and generation of free-radicals. Release of enzymes from neutrophil cytoplasmic granules and free oxygen radicals damage tissue, and play important roles in the pathogenesis of cattle shipping fever (Watson et al, 1995).

High-dose LKT lyses target cells. Experiments show that large plasma membrane defects are developed on the target cells and leakage of large intracellular molecules such as lactate dehydrogenase results (Clinkenbeard et al, 1989b). Deletion of LKT gene from *Pateurella haemolytica* bacteria results deletion of the leukolytic ability of bacterial culture supernatant (Murphy et al, 1995). LKT-induced cytolysis is extracellular calcium-dependent (Clinkenbeard et al, 1989). The important functions of LKT determine that LKT is a good immunogen of vaccine for cattle pneumonic pasteurellosis (reviewed by Confer, 1993).

LPS appears in most LKT preparations, and it is difficult to separate LPS from LKT (Yoo et al, 1995). Czuprynski and Welch (1995) proposed that LPS might be necessary for maximal production of some RTX toxin and might act as a cofactor in

some biological effects of RTX toxins. In the next chapter, the knowledge of LPS structure and function is reviewed.

Chapter III: Literature Review II

Lipopolysaccharide Structure and Function

1. Overview of Lipopolysaccharide

The cell wall of gram negative bacteria is composed of inner membrane (IM), periplasm and outer membrane (OM). IM is mainly composed of phospholipids. The periplasm has peptidoglycan, which form a hard coat around the cells. The OM has two leaflets. The main component of the inner leaflet is phospholipid, and the main component of the outer leaflet is lipopolysaccharide (LPS). There are many kinds of proteins, such as receptor, lipoproteins, porins inserted or attached to the cell coat. For *Escherichia coli*, LPS composed 4.9 μm^2 of the total 6.7 μm^2 bacterial surface. The remaining area is filled with proteins. There are about 3.5×10^6 molecules of LPS in a single bacterium (Nikaido and Vaara, 1987). As the term indicated, LPS consists of lipid and polysaccharide. It plays an important role in pathogenesis. LPS is a major virulence factor for septic shock and generalized inflammation (Rietschel and Brade, 1992). On the other hand, LPS is an immunomodulator that is capable of activating myeloid cells, increasing cell-mediated and humoral immune ability, and inducing nonspecific resistance to viral and bacterial infection (Alving, 1993). In the following paragraphs, the structure and biochemistry of LPS as well as the interaction between LPS and host cells will be discussed.

2. Structure of Lipopolysaccharide

LPS is an amphiphilic molecule. As Fig. 2.1 shows, LPS has a hydrophobic lipid A portion, a hydrophilic negatively-charged oligosaccharide core, and a hydrophilic polysaccharide O antigen.

Lipid A was named by Westphal and Luderitz in 1954 to distinguish it from the phospholipids. The backbone of lipid A is β-(1->6) interlinked biphosphorylated hexosamine (HexpN) disaccharide (Galanos et al, 1977). Four to six acyl chains, which are medium to long chain fatty acids or their hydoxyl derivatives, are linked to the backbone. The phosphate group at position 1 of HexpN (I) is α-anomer (Batley et al, 1982), which is essential for endotoxic activity (Ulmer, AJ, 1992). In the lipid A backbone, HexpN can be 2-amino-2-deoxy-D-glucose (GlcpN) and/or diaminohexose 2,3-diamino-2,3-dideoxy-D-glucopyranose (GlcpN3N or DAG). Three types of disaccharide out of four possible combinations have been found in nature. They are β-D-GlcpN-(1->6)-D-GlcpN, β-D-GlcpN3N-(1->6)-D-GlcpN, and β-D-GlcpN3N-(1->6)-D-GlcpN3N disaccharide. Among them, the form of GlcN3N-GlcpN is dominant (Moran, et al. 1991). There are usually four primary acyl groups, which are linked to the lipid A backbone directly, and two secondary acyl groups, which are linked to 3-hydroxyl group of primary acyl groups at Hex (II) (Erwin, et al. 1990). Two of primary acyl groups are ester-linked, and two of them are amide-linked. The fatty acids in LPS are saturated. Lipid A portion is responsible for LPS endotoxic activity.

The LPS core is divided into inner core and outer core. In the inner core, there are one to three unique 2-keto-3-deoxyoctonate (KDO), L-glycero-D-manno-heptopyranose

(LD-Hep) and/or D- glycero-D-mannoheptopyranose (DD-Hep). KDO acts as a bridge to connect lipid A to the core. The outer core is composed of hexose residues. Many kinds of hexoses including glucosamine (GlcN), D-glucose (Glc), N-acetylglucosamine (GlcNAc), D-galactose (Gal), and N-acetylgalactosamine (GalNAc) are found in the outer core. The sugar residues in the core may be phosphorylated by 2-aminoethyl phosphate groups (EtnP) or phosphate groups (Heinrichs et al, 1998). Although the core is not considered a virulence factor, it has been recently reported that LPS core may help pathogens to adhere to host cells (Jacques, 1996). The core is more conserved than the O-antigen, so that some researchers proposed using it as an immunogenic agent to produce antibodies against LPS (Stanislavsky et al. 1997).

The outermost part of LPS is O-antigen, which is composed of either repeating oligosaccharide units (heteropolymer) or one type of monosaccharide residual (homopolymer). Each repeating unit may have up to eight sugar residues (Skurnik et al, 1996). The repeating unit is very conserved, characteristic and unique for each bacterial strain, but the number of repeating unit in one single bacterium are variable. The typical range of the number of repeating units is 4-40 (Skurnik and Zhang, 1996). Therefore, O-antigen is the most variable part in the LPS.
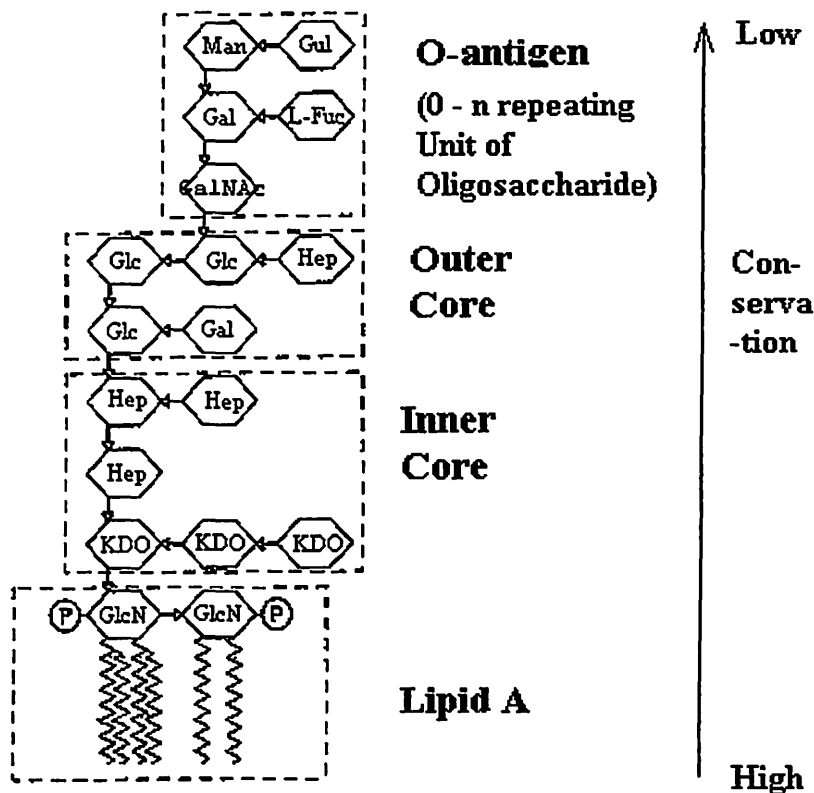
Fig. 2.1 Chemical structure of bacterial lipopolysaccharide (modified from Skurnik et al, 1996). Abbreviations: GlcN, glucosamine; P, phosphate; Glc, D-glucose; GlcNAc, N-acetylglucosamine; Gal, D-galactose; GalNAc, N-acetylgalactosamine; Hep, D(L)-glysero-D-mannoheptopyranose; Gul, D-gulose; KDO, 2-keto-3-deoxyotonic acid.

3. Biosynthesis and Function of Lipopolysaccharide

Like the synthesis of other carbohydrate polymers, LPS synthesis begins by activating sugars with ATP to form sugar-1-phosphate. The sugar-1-phosphate reacts with different nucleoside-triphosphates (NTP) determined by sugar type to form sugar-NDP, which is the active form of sugar for polymer synthesis. The core is synthesized coupled to lipid A. The synthesis of lipid A-core begins with assembling lipid A in the inner face of IM, the sugar residues are transferred to it one by one. After the synthesis is finished, lipid A-core is translocated to the outer side of inner membrane. For the synthesis of polysaccharide, if the O-antigen is homopolymer, then the sugar residues are transferred to the undecaprenol phosphate (unDp, a 55-carbon isoprenoid carrier lipid) and elongated on the cytoplasmic face of IM. If the O-antigen is heteropolymer, then the finished repeating units are linked by O-antigen polymerase (Wzy) into long chain on the unDp (Wzy-dependent pathway). The finished O-antigen is translocated into periplasmic side of IM by flippase (Liu et al, 1996), where the completed chains are translocated from Und-P to the proformed lipid A-core (Skurnik et al, 1996). The completed LPS is assemblied into outer leaflet of outer membrane.

As a major component of the OM, LPS is necessary for porin assembling in the outer membrane (Ried et al, 1990). Therefore, the intact LPS is very important to the stability of bacteria. As mentioned previously, lipid A is the most conserved and O-antigen is the most changeable. Mutations in lipid A usually are lethal, while the mutations in LPS core increase the permeability of outer membrane but are not lethal (Nikaido et al 1996). Bacteria with core mutation become more susceptible to attack by

leukocytes and macrophage (Hammond et al, 1984). The lipid A portion forms the outer leaflet of OM by hydrophobic interaction. The hydrophilic O-antigen protrudes out of OM. Therefore, LPS prevents small hydrophobic molecules from entering the periplasm, and large particles from reaching the bacteria surface. By this mechanism, the O-antigen part protects the bacteria from bactericidal effect, such as resistance to complement (Joiner et al, 1986) or phagocytosis (Stinavage et al, 1989). The LPS of OM is also responsible for resistance to some antibiotics (Rajyaguru et al, 1997).

As its name indicated, endotoxin is toxic to animals. Lipid A is responsible for endotoxin activity of LPS (Galanos et al, 1977). Evidence indicates that the number and location of acyl groups on the lipid A are important for endotoxin activity. For example, LPS from *Rhodobacter capsulatus* lacking two secondary acyl groups loses the ability to induce cytokines from target cells (Loppnow et al, 1990. Ulmer, Feist et al, 1992). Analysis of the LPS conformation indicates that the conformation of the LPS lacking the secondary acyl groups (Brandenburg et al, 1993) is different from the normal nonlamellar LPS conformation (Seydel et al. 1994). Therefore, Seydel and colleagues proposed that LPS or lipid A having lamellar structures is biological inactive, whereas LPS or lipid A forming nonlamellar structures is strongly endotoxic (Brandenburg et al, 1993, Seydel et al. 1994).

As a thymus-independent antigen, the O-antigen of LPS is strongly antigenic because it has highly repetitive structures. It crosslinks the surface immunoglobulins (sIg) on the mature B-lymphocyte surface and activates the B cells to produce polyclonal antibodies (Janeway and Travers, 1996). As an amphiphilic molecule, LPS tends to self-aggregate. Takayama and colleagues reported that the active form of LPS is the monomer

instead of aggregated LPS (Takayama et al, 1994). However, others reported an opposite

conclusion that LPS monomer acts as immunomodulator and aggregated LPS is more

toxic to host cells (Risco and Pinto, 1995).

4. Interaction of Lipopolysaccharide with Host Cells

LPS can activate macrophages, leukocytes, and endothelial cells. The interaction between host cells and LPS is mediated by several LPS-specific binding proteins. After activation, the physiology of host cells changes. In the following section, I will briefly discuss LPS recognition of molecules, LPS signal transduction pathways, and the cytokine networks.

4.1   LPS recognition molecules

mCD14 Cluster of differentiation antigen 14 (CD14) on the mature myeloid cells was first described during the First International Workshop on Human Leukocytes Differentiation Antigens. It was found that a panel of nine monoclonal antibodies (mAb) bound monocytes in human peripheral blood (Bernard and Boumsell, 1984). Later, the molecule recognized by these antibodies were demonstrated to be CD14 (Bazil et al, 1986a). This membrane bound CD14 molecule is named mCD14. The soluble form of CD14 molecule in the serum is named sCD14 (Bazil et al, 1986b). mCD14 is a 55 kilodalton (kDa) glycoprotein on mature macrophages, monocytes and polymorphonuclear leukocytes. It is a surface exposed protein, anchored to cell membrane by glycophosphatidylinositol (GPI, Haziot et al, 1988). There are about 50,000 CD14 molecules on one peripheral monocyte (van Voorhis et al, 1983) and 5,000 CD14 molecules on one neutrophil (Wright et al, 1991).

In 1990, Wright and his colleagues observed that anti-CD14 mAb inhibited the LPS-associated erythrocyte binding to macrophage. They also noticed that anti-CD14

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mAb inhibited LPS-induced TNF-α production from macrophage. Removal of mCD14 by phosphatidylinositol-specific phospholipase C decreased the LPS-associated erythrocyte binding (Wright et al, 1990a). Therefore, CD14 is proposed to be an LPS receptor on leukocytes. This concept is further proved by genetic techniques. Transfection of mCD14 gene into 70Z/3 pre-B cell line which does not express mCD14 enhanced the transfected cell sensitivity to LPS more than 1,000-fold (Lee et al, 1992). Transfection of mCD14 gene into LPS-insensitive chinese hamster ovary (CHO) cells produced LPS-responsive transgenic cells (Golenbock et al, 1993). Moreover, transgenic mice with human mCD14 become more sensitive to LPS (Ferrero et al, 1993). On the other hand, if mCD14 gene is knocked-out or disturbed, the LPS-sensitive cells become LPS-insensitive. For instance, mice with CD14 knockout are less responsive to LPS challenge (Haziot et al, 1996). LPS binding to mCD14 is facilitated by LPS-Binding Protein (LBP, Wright et al, 1990).

Therefore, mCD14 functions as LPS receptor on the myeloid cells. However, because 1) mCD14 is anchored to the cell membrane by GPI (Haziot et al, 1988), 2) it does not posses membrane-spanning domain (Haziot et al, 1988), and 3) replacing the GPI anchor with a transmembrane peptide domain does not alter the mCD14 function, some researchers proposed that mCD14 does not transduce the signal into target cells by itself (Ulevitch, 1995). How the mCD14 transduces a signal into the cells will be discussed later.

sCD14   sCD14 was first found in the cell culture supernatant, because of its ability to block anti-mCD14 mAb binding to monocytes (Maliszewski et al, 1985). On the SDS-PAGE, its molecular weight is smaller than mCD14. It is a 42 kDa single

peptide with intrachain disulfide bonds and an 11kD sialyated carbohydrate moiety (Bazil et al, 1986b). According to characters of sCD14, it is proposed that sCD14 is the enzyme-cleaved form of mCD14, though it has not been further demonstrated.

Like mCD14, sCD14 can bind LPS directly, and this binding can be enhanced by LBP (Haziot et al, 1993). By competing with mCD14 for LPS, sCD14 can prevent the oxidative burst in human monocytes induced by LPS in vitro (Schutt et al, 1991). However, sCD14 can not reduce the binding of LPS to monocytes in vivo (Rokita and Menzel, 1997). The main function of sCD14 is believed to be to facilitate LPS in activating mCD14-deficient cells such as endothelial cells (Haziot et al, 1993). Frey and his colleagues demonstrated that LPS activation of endothelial cells was serum-dependent. If the sCD14 in the serum is removed by mAb, then LPS can not activate endothelial cells. The endothelial cell responses are restored by addition of sCD14 to serum (Frey et al, 1992). The interaction between LPS-sCD14 and endothelial cells is reduced by soluble mediators from myeloid cell induced by mCD14-LPS interaction (Pugin et al, 1993). Evidence demonstrated that sCD14-LPS does not assist LPS-dependent activation of myeloid cells (Haziot et al, 1994).

The sCD14 may act as an LPS-binding reservoir, and thereby activate mCD14-deficient cells. When the endothelial cells are activated, the adhesion molecules on the endothelial cells are upregulated and many kinds of cytokines are synthesized and secreted, which lead to chemotaxis, cell adhesion, and coagulation.

LBP Lipopolysaccharide Binding Protein (LBP) is a serum glycoprotein protein. It has been found in human (Tobias et al, 1985), rabbits (Tobias et al. 1986), rats, mice, pigs, primates (Tobias and Ulevitc, 1993) and cattle (Khemlani et al, 1994). LBP is

conserved among species. For example, there is 69% homology between human LBP and rabbit LBP. In the blood, the normal level of LBP is about 0.5-10 μg/ml, however, it can be induced to the level of over 200 μg/ml during the acute infection phase (Tobias et al, 1992). LBP is a single peptide with molecular weight of 60 kDa. It has 452 amino acid residues and is post-translationally modified by attachment of several carbohydrate chains after being secreted (Schumann et al, 1990). LBP is synthesized mainly in hepatocytes according to the mRNA level detected by LBP probes (Tobias and Ulevitc, 1993).

As its name indicated, LBP specifically binds LPS with high affinity (Kirkland et al, 1993). The hydrophobic lipid A portion of LPS is the LBP binding site (Tobias et al, 1989). Both smooth and rough LPS bind to LBP with similar affinity of 10^{-9} (Mathison et al, 1992). Studies with truncated LBP indicates that the N-terminal 195 amino acids of LBP bind LPS in the similar manner to full-length LBP, however, the LPS bound to the truncated LBP can not be transferred to mCD14 (Han et al, 1994). Thus, LPS binding site of LBP is at N-terminal and CD14 stimulation site of LBP is at C-terminal.

LBP primarily functions as a lipid transfer protein. Pretreating macrophages with LBP does not enhance LPS uptake by macrophages, and macrophages do not bind erythrocytes coated with LBP (Wright et al, 1989). This means that LBP does not form stable complexes with mCD14. Macrophage can not recognize LBP coated-erythrocytes. However, if LPS is added, then macrophages can bind LBP (Wright et al, 1989). Therefore, LPS binding may induce LBP conformational change, which can be recognized by mCD14. In the presence of LBP, LPS is transferred to macrophages faster, and the ratio of the bound LPS molecules to mCD14 increased from about 1:1 to 20:1 (Hailman et al, 1994). Hailman and his colleagues proposed that LBP facilitates LPS

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binding to mCD14, but LBP is not consumed by the process (Hailman et al, 1994). Wurfel and his colleagues demonstrated that LBP also transfers LPS to high density lipoprotein (HDL), which lead to neutralization of LPS (Wurfel et al, 1994).

By facilitating LPS binding to mCD14, LBP enhances the sensitivity of myeloid cells to LPS. Addition of LBP to the system increases TNF-α production >250-fold (Schumann et al, 1990). Administration of LPS with anti-LBP antibodies protects the mice from lethal dose of LPS (Gallay et al, 1993). LBP-dependent LPS sensitivity enhancement can be eliminated by anti-CD14 mAb, which also suggests that LBP enhancement of LPS sensitivity is through mCD14, and mCD14 recognizes LBP-LPS complex (Wright et al, 1990a; Mathison et al, 1991). On the other hand, the created glycosylphosphatidylinositol-anchored form of LBP does not mediate cellular responses to LPS independently of CD14 (Tapping et al, 1999). This indicated that CD14 plays a specific role in mediating responses to LPS more than that of simply bringing LPS to the cell surface.

On the other hand, LBP may be an important protection component. After macrophages are treated with picomolar concentration of LPS in the presence of LBP, cells become resistant to the subsequent treatment of large dose of LPS (Mathison et al, 1993).

X-ray crystallography of *Limulus* anti-LPS factor (LALF) indicates that LPS-binding site of LALF is an amphiphilic loop (Hoess et al, 1993). Later, Schumann and his colleagues found that the LPS-binding domain is exchangeable between LPS-bind proteins such as LBP, LALF, bactericidal/permeability-increasing protein (Schumann et al, 1997). This indicated that LPS-binding proteins might share a common LPS-binding domain of amphiphilic loop.

CD18 CD18 is a subunit of β2 integrin. It is a single chain with molecular weight about 95kDa. There are three members of the β2 integrin, CD11a/CD18, CD11b/CD18 and CD11c/CD18. β2 integrin is a transmembrane receptor expressed on leukocytes only (Flaherty et al, 1997). One major function of β2 integrin is interacting with intercellular adhesion molecules (ICAM) on the endothelial cells and mediating migration of myeloid cells from the blood vessel to tissue. CD18 from chicken, human, pig, cattle, and mouse have been cloned and sequenced. Sequence alignment indicates that they share more than 80% homology (Fig. 2.2).

In 1986, it was reported that β2 integrin on leukocytes recognized LPS-coated erythrocytes and gram-negative bacteria. It is demonstrated that this interaction is mediated by CD18 and LPS (Wright and Jong, 1986). Wright and his colleagues also found that CD18-deficient cells produced normal amount of IL-1β and TNF-α when stimulated with LPS, and CD18-deficient cells show normal priming in response to LPS (Wright et al, 1990b). According to these results, they proposed that CD18 is not essential for cellar response. However, responses to LPS of 100ng/ml are observed for CD11b/CD18 genetic transfected CHO or fibroblasts which originally are LPS-nonresponsive cells (Flaherty et al, 1997). LBP also enhances the LPS-sensitivity of CD11/CD18-transfected CHO, thus implying that LBP can transfer LPS to CD11/CD18 (Ingalls et al, 1998). Bacteria or LPS binding to β2 integrin triggers phagocytosis, intracellular trafficking and killing. During this process, the cytokines are produced (Flaherty et al, 1997).

CD18 may also act as signal partners for other LPS receptors, and CD18 bound LPS can be transferred to CD14 (Todd and Petty, 1997). Deletion of intracellular cytoplasmic portion of integrin $\beta2$ does not disturb the LPS sensitivity of transgenic CHO (Ingalls et al, 1998). Thus, CD18, like mCD14, is an LPS binding protein, but not an signal transducer.

In vivo administration of anti-CD18 mAb prevents elevation of plasma TNF-α induced by LPS and prevents lethality by endotoxin shock. It is surprising that administration of anti-ICAM-1 mAb also prevents lethality. So the interaction between leukocytes and endothelial cells must be very important in endotoxin shock in vivo (Mukaida et al, 1996). The interaction between ICAM-1 and integrin $\beta2$ is also necessary for neutrophil adherence and aggregation (Wang et al, 1997).

LPS upregulates integrin $\beta2$ expression on granulocytes and monocytes (Wang et al, 1997. Blix et al, 1999). Recently, CD18 is proved to be a receptor of a bacterial exotoxin named *Pasteurella haemolytica* leukotoxin, one of Repeat-in-Toxin (RTX) toxin (Li and Clinkenbeard, 1999). Though both RTX toxin and LPS bind CD18, no inhibition of each other has been reported. LPS and RTX toxin may bind at different sites. It is interesting to note that there is no specificity for LPS binding to CD18, while there is species-specificity for RTX toxin binding to CD18. Whether there is synergism between exotoxin and endotoxin and how the two toxins bind to CD18 need further investigation.

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  1 MPRDCCLWLPAMTWVLLLLT TAFAAECPKIKVG TCRNCIQSGPGCAWCKK
  1 MLCRCS-PLLLLVGLLTLR- SALSQECAKYKVS TCRDCIESGPGCAWCQK
  1 MLGLRPSLLLALAGLFFLG- SAVSQECTKYKVS SCRDCIQSGPGCSWCQK
  1 MLGLRP-PLLALVGLLSLG- CVLSQECTKFKVS SCRECIESGPGCTWCQK
  1 MLRQRP-QLLLLAGLLALQ- SVLSQECTNYKVS TCRDCIESGPGCAWCQK
       L  RP   LAl GlL      SALsQ TKY  St rd  e     A Q

 51 LSFTKAGEPDSNRCD TIEQLQQRGCP ENEIEFP VNEIKRTQDSAFSNKIQ
 49 LNFSGQGEPDSVRCD TREQLLAKGCV ADDIVDP R-SLAE TQEDQAGGQKQ
 50 LNFTGPGEPDSLRCD TRAQLLLKGCP ADDIMDP R-SIANPEFDQRGQRKQ
 49 LNFTGPGDPDSIRCD TRPQLLMRGCA ADDIMDP T-SLAE TQEDHNGGQKQ
 49 LNFTGQGEPDSIRCD TRAELLSKGCP ADDIMEP K-SLAE TRDSQAGSRKQ
       N tG  e         R q L k PAdd MD    S1AETQ DQ G  K

101 LTPQEVHLKLRIREPAEFDVKFRRATGYPIDIYYLMDLSYSMLDDLENVK
 98 LSPQKVTLYLRPGQAATFNVTFRRAKGYPIDLYYLMDLSYSMLDDLINVK
 99 LSPQKVTLYLRPGQAAAFNVTFRRAKGYPIDLYYLMDLSYSMLDDLNNVK
 98 LSPQKVTLYLRPGQAAAFNVTFRRAKGYPIDLYYLMDLSYSMLDDLRNVK
 98 LSPQEVTLYLRPGQAVAFNVTFRRAKGYPIDLYYLMDLSYSMVDDLVNVK
       s  KTY  PGqAAA n T    K          l          l

151 KLGGQLLRALESTTPSRRIGFGSFVDKTVLPFVNTHPEKLKNPCPNKDSN
148 KLGGDLLRALNEITESGRIGFGSFVDKTVLPFVNTHPEKLRNPCPNKEKE
149 KLGGDLLQALNEITESGRIGFGSFVDKTVLPFVNTHPEKLRNPCPNKEKA
148 KLGGDLLRALNEITESGRIGFGSFVDKTVLPFVNTHPDKLRNPCPNKEKE
148 KLGGDLLRALNGITESGRIGFGSFVDKTVLPFVNTHPEKLRNPCPNKEKE
        D R NEI E G               e   r       eKE

201 CQPPFAFKHILSLTDNAEKFESEVGKQFISGNLDAPEG-LDAMMQAAVCG
198 CQAPFAFRHVLKLTDNSNQFQTEVGKQLISGNLDAPEGGLDAMMQVAACP
199 CQPPFAFRHVLKLTDNSNQFQTEVGKQLISGNLDAPEGGLDAIMQVAACP
198 CQPPFAFRHVLKLTNNSNQFQTEVGKQLISGNLDAPEGGLDAMMQVAACP
198 CQPPFAFRHVLKLTDNSKQFETEVGKQLISGNLDAPEGGLDAMMQVAACP
        P   r v K d sNQ qt      L         G   m  VAP

250 DLIGWRNVTRLLVYATDDGFHFAGDGKLGGILTPNDGQCHLEDNMYKKSN
248 EEIGWRNVTRLLVFATDDGFHFAGDGKLGAILTPNDGRCHLEDNLYKSSN
249 EEIGWRNVTRLLVFATDDGFHFAGDGKLGAILTPNDGRCHLEDNMYKRSN
248 EEIGWRNVTRLLVFATDDGFHFAGDGKLGAILTPNDGRCHLEDNLYKRSN
248 EEIGWRNVTRLLVFATDDGFHFAGDGKLGAILTPNDGRCHLEDNLYKSSN
     eE            f          a     R    l

300 EFDYPSVGQLVQKLAENNIQPIFAVTSKMVDVYKKLSDMIPKSAVGELNE
298 EFDYPSVGQLAHKLAESNIQPIFAVTKKMVKTYEKLTDIIPKSAVGELSE
299 EFDYPSVGQLAHKLSESNIQPIFAVTKKMVKTYEKLTEIIPKSAVGELSD
298 EFDYPSVGQLAHKLAENNIQPIFAVTSRMVKTYEKLTEIIPKSAVGELSE
298 EFDYPSVGQLAHKLAESNIQPIFAVTKKMVKTYEKLTEIIPKSAVGELSE
              AH a S        Kk KT E tei          Se

350 DSSNIIELIQVAYNNLSSRIILDHSTLPDVLDVKYDSICNNNTGAKNEAR
348 DSSNVLELIKNAYNKLSSRVFLDHNALPDTLKVTYDSFCSNGVSQVNQPR
349 DSSNVVQLIKNAYYKLSSRVFLDHSTLPDTLKVTYDSFCSNGASSIGKSR
348 DSSNVVHLIKNAYNKLSSRVFLDHNALPDTLKVTYDSFCSNGVTHRNQPR
348 DSRNVVELIKNAYNKLSSRVFLDHSTLPDTLKVTYDSFCSNGKSQVDQPR
        S vvE KN NK   vF  ST    TKT    F SGS   NQP
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400  GQCDNVKINDEVTFKVKVTANECIKSQSFTIRPLGFTDTLTVHLDSICDC
398  GDCDGVQINVPITFQVKVTASECIQEQSFVIRALGFTDTVTVRVLPQCEC
399  GDCDGVQINNPVTFQVKVMASECIQEQSFVIRALGFTDTVTVQVRPQCEC
398  GDCDGVQINVPITFQVKVTATECIQEQSFVIRALGFTDIVTVQVLPQCEC
398  GDCDGVQINVPITFQVKVTATECIQQQSFTIRALGFTDTVTVRVLPQCEC
        D  G Q  VPi Q   T    QE  V A    Tv  vLPQ e

450  DCREQP-DPTACSGNGKVVCGICSCNLSYTGKNCECDTKGKTSKELEGSC
448  RCGDSSKERTLCGNKGSMECGVCRCDAGYIGKHCECQTQGRSSQELEGSC
449  QCRDQSREQSLCGGKGVMECGICRCESGYIGKNCECQTQGRSSQELERNC
448  RCRDQSRDRSLCHGKGFLECGICRCDTGYIGKNCECQTQGRSSQELEGSC
448  QCRDASRDGSICGGRGSMECGVCRCDAGYIGKNCECQTQGRSSQELEGSC
        RdQSRd sL GGK mE  iR d GI N    QQ rs Q   GS

499  RKDNSSVICSGLGDCVCGQCVCHTSDVPGKEIYGTFCDCDNMNCEFHNGS
498  RKDNSSIICSGLGDCICGQCVCHTSDVPNKKIYGQFCECDNMNCERFDGQ
499  RKDNSSIVCSGLGDCICGQCVCHTSDVPNKEIFGQVCECDNVNCERYNSQ
498  RKDNNSIICSGLGDCVCGQCLCHTSDVPGKLIYGQYCECDTINCERYNGQ
498  RKDNSSIICSGLGDCICGQCVCHTSDVPNKKIYGQFCECDNVNCERYDGQ
        S ii        i   v       N  y Qf e N    RYnGQ

549  LCGGEERGRCDCGECKCTPKYEGSACQCKKSTDGCRNSRQNECSLRGSCP
548  VCGGEKRGLCFCSTCRCQEGFEGSACQCLKSTQGCLNLQGVECSGRGRCR
549  VCGGSDRGSCNCGKCSCKPGYEGSACQCQRSTTGCLNARLVECSGRGHCQ
548  VCGGPGRGLCFCGKCRCHPGFEGSACQCERTTEGCLNPRRVECSGRGRCR
548  VCGGEKRGLCFCGTCRCDEQYEGSACQCLKSTQGCLNLDGVECSGRGRCR
        v  E  L F G  R  PGy      ks   L  R V  G  R R

599  CNRCQCRGGYQPPFCEECPGCPSPCGR-HISCVECKSFNSGPLAKNCSVA
598  CNVCQCDFGYQPPLCTDCPSCQVPCAR-YAKCAECLKFDTGPFAKNCSAE
599  CNRCICDEGYQPPMCEDCPSCGSHCRDNHTSCAECLKFDKGPFEKNCSVQ
598  CNVCECHSGYQLPLCQECPGCPSPCGK-YISCAECLKFEKGPFGKNCSAA
598  CNVCQCDPGYQPPLCSECPGCPVPCAG-FAPCTECLKFDKGPFAKNCSAA
        V Q D    P L e G PSP     S A LK dK  FA      AA

648  CTSIQLADEPRAGSRQCKEKDSENCWISFYMAQDDGEEMYTVTVDPKKEC
647  CGTTKLLPSRMSG-RKCNERDSEGCWMTYFLVQRDGRDNYDLHVEETREC
649  CAGMTLQTIPLKK-KPCKERDSEGCWITYTLQQKDGRNIYNIHVEDSLEC
647  CPGLQLSNNPVKG-RTCKERDSEGCWVAYTLEQQDGMDRYLIYVDESREC
647  CGQTKLLSSPVPG-RKCKERDSEGCWMTYTLVQRDGDRYDVHVDDMLEC
          P  Gr  K r  G   TvTl    Rd   H d

698  PEPPNIALIVGSTIAGVALIGLLLLLTWRLLTEIFDRREYRRFEKEKSKA
696  VKGPNIAAIVGGTVGGVVLVGIFLLVIWKVLTHLSDLREYKRFEKEKLKS
698  VKGPNVAAIVGGTVVGVVLIGVLLLVIWKALTHLTDLREYRRFEKEKLKS
696  VAGPNIAAIVGGTVAGIVLIGILLLVIWKALIHLSDLREYRRFEKEKLKS
696  VKGPNIAAIVGGTVGGVVLVGILLLVIWKALTHLSDLREYHRFEKEKLKS
        VKG  iA   G v  vV i iL  vI kA TH1S L  r     L s

748  KWNEADNPLFKSATTTVMNPRFDGQ
746  QWNN-DNPLFKSATTTVMNPKFAER
748  QWNN-DNPLFKSATTTVMNPKFAES
748  QWNN-DNPLFKSATTTVMNPKFAES
746  QWNN-DNPLFKSATTTVMNPKFAES
        Q  N           k AES
```

35

Fig. 2.2. Multiple protein sequence alignment of CD18 from various species by NCSA Computational Biology Program (Hofmann and Baron, 1997). From top to bottom, the species is chicken, pig, mouse, human, bovine. The region of the bovine CD18 are listed following: 1-22 is signal peptide, 23-670: extracellular portion.701-723: transmembrane domain. 724-769: intracellular domain.

Toll-like receptor-2 In 1998, Yang and his colleagues reported that Toll-like receptor-2 (TLR2) mediated LPS-induced cellular response (Yang et al, 1998). Protein sequence alignment indicated that the intracellular region of TLR2 is homologous with the intracellular portion of interleukin (IL)-1 receptor. This homologous portion of IL-1 receptor is believed to activate IL-1-receptor-associated kinase. On the other hand, TLR2-mediating LPS response is dependent on LBP and enhanced by mCD14 (Yang et al, 1998). Therefore, they proposed that TLR2 is the direct mediator in signal-transducing process. Other lab observed that the Toll-like receptor-4 (TLR4) deficient mice were hyporesponsive to LPS (Hoshino et al, 1999). If HEK 293 cells were transfected with TLR4 gene, the cells became responsive to LPS in dose- and time-dependent manner (Chow et al, 1999). These evidences indicated that TLR4 might also regulate LPS response.

Other LPS receptor CD18, mCD14 have been proved to be LPS receptor on cell surface. But in some circumstance, blocking them with mAb cannot prevent macrophage activation by low concentration of LPS (Jungi et al, 1997), therefore, other LPS receptors must exist.

Lei and Morrison reported that a cell surface 73kDa protein (p73) was identified as LPS binding protein by photoactivatable LPS derivatives (Lei and Morrison, 1988). The lipid A, rough LPS or smooth LPS bound to p73 in similar manner, which indicates that p73 recognizes lipid A portion of LPS (Lei et al, 1991). However, the distribution of p73 is wide, and it is expressed on many type of cells such as murine splenocytes (Lei and Morrison, 1988), rat trophoblast cells (Hunt et al, 1989), and human peripheral blood cells (Halling et al, 1992). In contrast, the distribution of CD14 is of narrow scale of LPS

target cells such as monocytes, macrophage and neutrophils. Though the distribution of p73 is intriguing, that anti-73kDa protein antibody (mAb 5D3) inhibits LPS-induced macrophage response indicates that p73 may play an important role in LPS-induced signal transduction (Chen et al, 1990).

Additional LPS binding proteins of 38kDa (Lei et al, 1993), 18kDa and 25kDa (Kirkland et al, 1990), 65kDa and 55kDa (Hara-Kuge et al, 1990) have been identified. Among them, the 38kDa protein is interesting, because it binds both rough and smooth LPS but does not bind lipid A. This indicates that p38 may specifically recognize LPS inner core (Lei et al, 1993). High density lipoprotein (LDH) also can bind LPS, and neutralize LPS activity.

The LPS recognition molecules are summarized in Fig. 2.3.
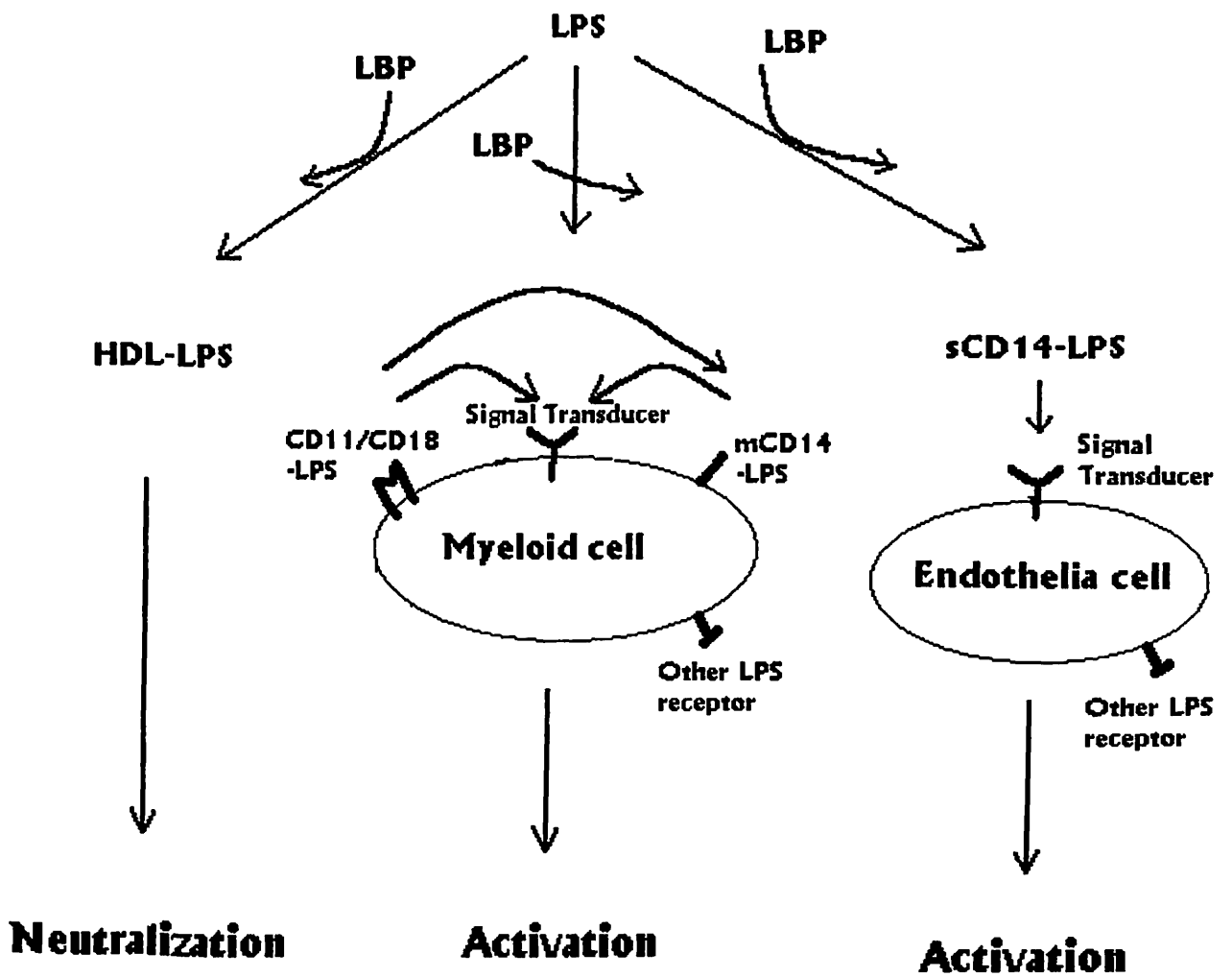
Fig. 2.3 LPS recognition proteins (modified from Schletter et al, 1995)

4.2   LPS signal transduction pathway

LPS-dependent activation of myeloid cells can be eliminated by anti-mCD14 mAb at low level of LPS. However at high LPS concentration, LPS can activate macrophages regardless of the amount of anti-mCD14 mAb added. These results suggest there are CD14-dependent and CD14-independent pathways.

As described previously, mCD14 is a GPI-anchored protein. If a transmembrane polypeptide is used to replace GPI, the transgenic cells can still be activated by LPS in a similar manner (Pugin et al, 1998). Therefore, GPI-anchoring is not required for LPS-mediated myeloid cell activation. How does CD14 transduce signal into cells? By analogy to other GPI-anchored receptors (Brown 1993), a transmembrane protein is proposed to be required to bridge the LPS-mCD14 to the intracellular signal system. This signal transduction protein may serve as subunit of LPS receptor or as a separate protein to recognize LPS binding mCD14 (Ulevitch and Tobias, 1995). Although this essential protein has not been discovered, some evidence supports this hypothesis. Sequence alignment of CD14 with known proteins found that a repeating leucine-rich motif, which is known for promoting protein-protein interaction, was found in CD14 (Ferrero et al 1990).

Several GPI-anchored cell-surface proteins, such as CD14, CD24, CD48, CD55 and CD59, have been proved to associate with protein tyrosine kinases (Stefanova et al, 1991). As we know, protein tyrosine kinases are important components in signal transduction.   This association suggests a potential signal transduction pathway. Experiment results also prove that protein tyrosine kinase and phosphatase are actually

involved in the LPS-mediated activation of myeloid cells (Beaty et al, 1994). Beaty and his colleagues (1996) reported that addition of LPS increased the activity of two protein tyrosine kinases, Hck and Lyn in macrophages. In the presence of protein tyrosine kinase inhibitors herbimycin A or genistein, macrophages produce less TNF-α and IL-6 when stimulated by LPS, and in the presence of tyrosine phosphatase inhibitor sodium orthovanadate, macrophages produce more TNF-α (Beaty et al, 1994). Protein tyrosine kinase activation by LPS is due to the autophosphorylation of Hck and Lyn, two members of src tyrosine kinase family members (Henricson et al, 1995). Recently, Marie and his colleagues reported that mitogen-activated protein kinase (MAPK) pathway is also involved in LPS-induced IL-8 production from human monocytes and polymorphonuclear cells. Inhibition of the kinases in MAPK pathway blocks the production of IL-8 (Marie et al, 1999). LPS can activate nuclear factor κB (NF–κB) in mCD14-transfected chinese hamster ovary fibroblasts (Golenbock et al, 1993). NF-κB is a member of rel family. It localizes in the nucleus, binds DNA and activates RNA transcription (Ruben et al, 1992; Baeuerle and Henkel, 1994). Although the details of signal transduction are still obscure, the event sequence of CD14-dependent cell activation by LPS may be LBP-LPS → mCD14-LPS → signal transducer → protein tyrosine activation → MAPK cascade activation→ NF-κB→mRNA transcription →protein expression.

Ingalls and his colleagues found that deletion of integrin β2 intracellular portion does not affect the LPS-sensitivity of CD11/CD18-transfected CHO cells. The response of integrin β2-tranfected CHO cell to synthetic lipid A analogues is the same as that of

mCD14-transfected CHO cells (Ingalls et al, 1998). Therefore, CD11/CD18 and CD14 may have the same LPS signal pathway.

LPS can enter the cells and directly interact with intracellular components. Uptake of LPS by cells may occur specifically and/or nonspecifically. For example, LPS-CD18 triggers the internalization of LPS (Flaherty et al, 1997). LPS- mCD14 mediates rapid uptake of LPS into neutrophils (Gallay et al 1993). "Immunogold electron microscope analysis found that mCD14 bound LPS moved principally into noncoated structure such as tubular invagination, intracellular tubules and vacuoles, whereas little moved into coated pits and vesicles" (Kitchens et al, 1998). Aggregation increases LPS internalization through noncoated pathway (Kitchens et al, 1998). It is proposed that LPS aggregates are toxic, while LPS monomers are immuno-stimulators which enhances the monocytes or macrophages killing ability (Risco and Pinto, 1995). Immunogold electron microscope analysis also indicates LPS moves from plasma membrane to cytoplasm, phagosomes, mitochondria to nuclear membrane, and finally reaches nucleus as the incubation time prolonging in endothelial cells (Risco and Pinto, 1995).

As mentioned above, LPS can also activate CD14-deficient cells, such as CD18-transfected CHO cells (Flaherty et al, 1997), Kupffer cells (Lichman et al, 1998) or endothelial cells. The mechanism is not yet known. It is possible that after LPS enters cells, it interacts with intracellular components directly. Although most of intracellular LPS are destroyed by enzymes (Gallay et al 1993), the remaining LPS can interact with many cell components. Lipid A is able to bind protein kinase C (PLC) directly and activate PLC (Ellis et al, 1987). It is also proved that LPS can directly interact with tubulin and microtubule associated proteins and activate protein kinase cascade (Risco et

al, 1993). LPS inhibits polymerization of microtubules at high concentration (250μg/ml), and causes partial inhibition of microtubule polymerization at lower concentration (Risco and Pinto, 1995). Taxol, a β-tubulin interaction drug, can mimic some actions of LPS (Manthey et al, 1992). This disruption of microtubular network by LPS may play an important role in the process of endothelial cell degeneration, and lead to capillary permeability increase. At high concentration, LPS may enter the cells, activate PLC, initiate a cytoskeleton associated signal cascade, and/or directly interact with nucleic acids, so that gene regulation is affected.

4.3 Cytokines induced by LPS

LPS induces transcription of several genes, thereby altering gene expression. These gene products include cytokines (Matsukawa et al, 1998), adhesive proteins (Tonnesen MG. 1989) and enzymes which produce low molecular weight proinflammatory mediators. The LPS-induced cytokines include tumor necrosis factor alpha (TNF-α), interleukin-1 (IL-1), IL-1 receptor antagonist (IL-1ra), IL-8 (Matsukawa et al, 1998), IL-10, IL-12, IL-6 (Wu et al, 1999) etc. The adhesion molecules include ICAM-1, integrins etc (Tonnesen MG. 1989, Spittler et al, 1999).

Among the cytokines, INF-α, IL-1, IL-6 and IL-8 are regarded as pro-inflammation factors, because they induce inflammation (Dinarello, 1994). IL-1ra, IL-10 are regarded as anti-inflammation factors, because they downplay inflammation (Henderson and Bodmer, 1996). It is well established that pro-inflammation cytokines are necessary for host to battle against the bacterial infections. The anti-inflammation

cytokines are also necessary for host to survive from infections. For example, the IL-10 gene knockout mice die of the response to the normal commensal flora because the host loses the ability to control the inflammation (Kuhn et al, 1993). These cytokines affect (up- or down-regulate) each other to form a cytokine network defined as "complex interaction of cytokines by which they induce or suppress their own synthesis or that of other cytokines or their receptors, and antagonize and synergize with each other in many different ways" (Ibelgaufts, 1995). Two adhesion molecules, endothelial-leukocyte adhesion molecule-1 and intercellular adhesion molecule-1 (ICAM-1), are upregulated at transcriptional level by LPS and some cytokines (Tonnesen, 1989). As described previously, interaction between ICAM-1 and CD11/CD18 plays key role for migration of neutrophils to infection sites in response to chemotactic factors. As indicated earlier, inhibition of myeloid cell migration by mAb decreases lethality of septic shock (Mukaida et al, 1996). The cytokines also act on endothelial cells to increase the permeability of blood vessels. The accumulation of fluid in the tissues leads hypotension, multiorgan failure and death.

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Chapter IV: Summary and problem statement

The literature review indicates that LKT has an extensive role in the pathogenesis of cattle shipping fever. In contrast to other members of the RTX (Repeat-in-toxin) toxins family, LKT has a narrower target cell specificity. LKT recognition of its target cells is one of the key steps in the LKT function. Understanding the mechanism of LKT recognition of host cells might be helpful in control of the disease.

Therefore, one of the objectives of this study was to identification of LKT receptor. After trying several methods, finally, the species-specific band was observed by LKT ligand blot procedure. This specific band was then identified as CD18, a subunit of β-2 integrin. We noticed that pro-LKT recognized this band too. Therefore, LKT function on host cells may have several critical steps besides receptor binding.

In vivo, LPS has obvious effects on hosts. Large dose of LPS induces septic shock, while small amount of LPS can prime host immune defense cells, which may become more susceptible to LKT. LPS priming the cell may be through its direct interaction with host cells or indirectly through cytokine action. In order to understand the mechanism of LPS and LKT in the pathogenesis, it is necessary to obtain purified LKT and LPS. However, in vitro, LKT and LPS are tightly associated with each other, and it is very difficult to get LPS-free LKT. Is this association critical for LKT function? Do LKT and LPS act synergistically?

Thus, the second objective of this study was to identify the relations between LKT and LPS. In the process of purification of LKT, the difficulty of separating LPS from LKT was experienced. It was found that the LPS-free LKT was still active and remained

its target cell specificity. Therefore, LPS is not necessary for LKT function. However, leukolytic activity of LPS-free LKT was less stable. Addition of LPS back to LPS-free LKT enhanced LKT stability and activity. LPS•LKT complex was also detected.

To examine whether there is synergism between LPS and LKT, sublytic CCS LKT, LPS-free LKT, isolated LPS and LKT plus LPS were used to activate bovine lymphoma cells. The TNF-α mRNA transcription level was used to quantitate the cell response. It was found that bovine lymphoma cells were more responsive to LKT than to LPS. Flow cytometry proved that BL3 cells was CD18 positive and CD14 negative. As expected, anti-CD18 antibody inhibited LKT activity, but surprisingly, enhanced LPS activity on BL3 cells. No synergism was observed between sublytic LKT and LPS.

In summary, LPS may play a role in stabilizing LKT structure and increasing LKT leukolytic activity. However, LPS is not necessary for LKT function or target cell specificity. LKT specifically recognizes CD18 that mediates species-specific actions. These actions include leukolysis and induction of cytokine expression. Although LKT complexes with LPS, synergism between sublytic LKT and LPS in inducing TNF-α expression on bovine lymphoma cells does not occur.

* Chapter V:

Bovine CD18 identified as a species specific receptor for

Pasteurella haemolytica leukotoxin

ABSTRACT

Ligand blotting of _Pasteurella haemolytica_ leukotoxin (LKT) susceptible BL3 bovine lymphoma cell membranes with LKT detected two putative receptors with M_r of 95 and 100 kDa, whereas no LKT binding to membrane proteins was detected for LKT non-susceptible human leukemic cells. Anti-bovine CD18 and CD11a/CD18 mAb recognized 95 and 100 kDa bands from BL3 cell membranes. CD18 isolated from BL3 cell membranes bound LKT. Pre-incubation of BL3 cells with anti-bovine CD18 or CD11a/CD18 mAb caused partial inhibition of LKT-induced leukolysis. Therefore, we propose that bovine CD18 acts as a species-specific leukocyte receptor for P. haemolytica LKT.

Keywords: _Pasteurella haemolytica_, leukotoxin, leukocytes, CD18, toxin receptor, ligand blotting.

1. Introduction

The Gram-negative bacterial "repeats in toxins" (RTX) family of pore-forming cytolysins exhibit extensive sequence homology and similarities in gene arrangement, mechanisms of pro-toxin activation, toxin secretion, and target cell intoxication (Welch et al., 1996). Most of the RTX toxins have broad target cell specificity, but two of the RTX toxins, Actinobacillus actinomycetemcomitans leukotoxin (LTX) and *Pasteurella haemolytica* leukotoxin (LKT) are specific for human and bovine leukocytes, respectively (Tsai et al., 1979; Shewen and Wilkie, 1982). Specific receptor binding is hypothesized to mediate this target cell specificity. Recently, a β_2 integrin composed of 150 kDa CD11a and 100 kDa CD18 was identified as a specific receptor on HL-60 human myelomonocytic leukemic cells for two RTX toxins, A. actinomycetemcomitans LTX and Escherichia coli α-hemolysin (Lally et al., 1997). In light of these findings, we used ligand blotting to examine whether a similar β_2 integrin on bovine leukocytes is a receptor for the RTX toxin P. haemolytica LKT.

2. Materials and methods

2.1 Preparation of P. haemolytica LKT.

LKT preparations were late-logarithmic phase 60% ammonium sulfate concentrated culture supernatants produced in RPMI-1640 medium from a wildtype strain and strain SH1562 containing a non-polar insertion in the lktC gene which produces inactive pro-LKT (Fedorova and Highlander, 1997; Sun and Clinkenbeard, 1998). The concentrated culture supernatants were dialyzed against phosphate buffered saline (PBS), pH 7.2 and contained 1.5 mg protein/ml.

2.2 Cultivation of tissue culture cells and preparation of cell membranes

Tissue culture cells (BL3, Raji and HL60) were obtained from and cultured as described by American Type Culture Collection, Rockville, MD, USA. Isolated membranes were prepared from 10^9 PBS-washed tissue culture cells suspended in 20 ml of 50 mM 3-(N-morpholino)-propanesulfonate, pH 7.0 (MOPS buffer), which contained one protease inhibitor cocktail tablet (Boehringer Mannheim Corp., Indianapolis, IN, USA), and subjected to 10-passes of a Potter-Elvenhjem tissue grinder with a 0.1 mm clearance. Unbroken cells and nuclei were removed by centrifugation at 1,400 xg for 15 min. The membranes were then collected by centrifugation at 25,000 xg for 45 min and washed in MOPS buffer twice. The membranes were extracted with 1% Triton X100 in MOPS buffer for 30 min at 4°C.

2.3 Ligand blotting

Ligand blotting was conducted on Triton X100 membrane extracts by mixing 1:1 (v/v) with non-reducing SDS-PAGE sample buffer and subjected to SDS-PAGE on 10% gels (Naglich, et al., 1992). Following transfer to nitrocellulose membranes, the

membranes were blocked with 1% gelatin, washed with PBS containing 0.15% Tween 20 and 2 mM CaCl$_2$ (PBSTC) twice. The membranes were then incubated with the 10 μg/ml LKT in blotting buffer (0.5% Triton X100, 75 mM NaCl, 5 mM CaCl$_2$, 0.67% gelatin, 5 mM Tris, pH 8.0) overnight. The nitrocellulose membranes were washed 3X with PBSTC and incubated sequentially with 1:400 murine anti-LKT mAb C6 (Murphy, et al., 1995) for 8 hours, 1:1,000 biotin conjugated goat anti-mouse IgG (Sigma Chemical Co., St. Louis, MO, USA) for 8 hours, and 1:3,000 streptavidin-alkaline phosphatase conjugate (Bio-Rad Laboratories, Hercules, CA, USA) for 4 hours, with washing with PBSTC 3X between each step. Bound alkaline phosphatase was developed with BCIP/NBT reagent (Sigma Chemical Co.).

2.4 Immunoprecipitation of CD18

Murine anti-bovine CD11a/CD18 (BAT75A and MUC76A) and CD18 (BAQ30A) mAb were obtained from VMRD, Pullman, WA, USA. CD18 was isolated by immunoprecipitation from 0.5 ml extract of BL3 cell membranes containing 0.75 mg protein by incubation with 50 μg BAQ30A or control buffer for 2 hours at 4°C followed by incubation with 50 μl Protein-A agarose (Bio-Rad Laboratories) for 2 hours. Unbound CD18 was collected as a supernatant, and the gel washed 4-times with 1.0 ml blotting buffer. Bound CD18 was eluted with 150 μl 100 mM glycine, pH 2.7.

2.5 Inhibition of LKT-induced leukolysis by anti-β$_2$ integrin mAb

Inhibitory effect of pre-incubation of 2X10^5 BL3 cells with 10 μg anti-bovine CD11a/CD18 or CD18 mAb in 90 μl RPMI-1640 for 45 min at 37°C on LKT leukolytic activity was examined. Leukolytic activity was assayed by addition of 100 ng LKT in 10 μl to the pre-incubated cells followed by an additional 45 min incubation at 37°C.

Exposed cells were cooled on ice and mixed with 100 μl 0.4% trypan blue, and viable cells were counted in triplicate using a hemocytometer. The counts were normalized to the negative control, RPMI-1640 with 10 μg irrelevant murine mAb MOPC-21 (Sigma Chemical Co.) and no LKT.

3. Results

LKT ligand blotting of LKT susceptible BL3 bovine lymphoma cell membranes detected two putative receptors with M_r of 95 and 100 kDa (Fig. 1A, lane 1). A $\approx$70 kDa band was observed in blots of the LKT non-susceptible human Raji lymphoma cell membranes both in the presence and absence of LKT (Fig. 1A and 1B, Lane 2), suggesting that this band was alkaline phosphatase. No bands were detected for LKT ligand blotting of the LKT non-susceptible human HL60 myelomonocytic leukemic cells (data not shown). Ligand blotting with the non-acylated, inactive pro-LKT also identified the 95 and 100 kDa bands in BL3 cell membranes, but none were detected by pro-LKT in the Raji or HL60 cell membranes (data not shown).

Immunoblotting of the BL3 cell membranes with a murine anti-bovine CD18 (BAQ30A) or CD11a/CD18 (BAT75A) mAb also detected bands of 95 and 100 kDa (Fig. 2A and B, lane 1). An anti-bovine CD18 (BAQ30A), but not an anti-bovine CD11a/CD18 (BAT75A), recognized a 100 kDa band as well as two bands with slightly higher M_r on Raji cell membranes (Fig. 2A and B, lane 2), suggesting that BAQ30A has some cross-reactivity with human CD18. The $\approx$70 kDa putative alkaline phosphatase band was observed on blots of both BL3 cell and Raji cell membranes. No bovine CD18 or CD11a/CD18 immunoreactive bands were detected on HL60 cells (data not shown). One anti-bovine CD11a/CD18 mAb (MUC76A) did not recognize CD18 bands on blots of BL3, Raji, or HL60 cell membranes.

The CD18 from BL3 cell membranes was isolated by immunoprecipitation using BAQ30A. As shown in Fig. 3, lane 4, the isolated CD18 was confirmed to react with LKT by ligand blotting. In the absence of BAQ30A in the immunoprecipitation

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procedure, no LKT reaction was detected by ligand blotting for the protein elutant (Fig. 3, lane 2).

Pre-incubation of BL3 cells with the anti-bovine CD18 (BAQ30A) or the anti-bovine CD18/CD11a (BAT75A) that reacted with the 95 and 100 kDa bands on immunoblots of the BL3 cells membranes caused partial inhibition of LKT-induced leukolysis, but the non-immunoreactive anti-bovine CD11a/CD18 (MUC76A) did not inhibit LKT-induced leukolysis (Table 1). The inhibition of LKT-induced leukolysis of BL3 cells was dependent on the mAb concentration (data not shown).

4. Discussion

The RTX toxin family consists of a related group of bacterial toxins with broad target cell susceptibility, except for two RTX toxins which are specific for leukocytes from host species for which their parent bacterial species are pathogens. It has been hypothesized that the narrow target cell specificity of the RTX leukotoxins is mediated by species-specific leukocyte receptors present only on the susceptible leukocytes, but absent from non-susceptible, non-leukocytic cells. This hypothesis was partially substantiated by work of Lally and colleagues (1997), who demonstrated that A. actinomycetemcomitans LTX leukolytic activity was blocked by a mAb which recognized CD11a/CD18 from susceptible human HL60 leukemic cells. Transfection of LTX non-susceptible human K562 erythroleukemic cells with the human CD11a and CD18 genes converted these cells to LTX susceptible cells.

We extend this work to demonstrate that bovine CD18 is a receptor for the other RTX leukotoxin, P. haemolytica LKT. The LKT-binding domains of bovine CD18 are apparently species-specific to bovine CD18, because LKT did not recognize human CD18. Furthermore, inactive pro-LKT (Fedorova and Highlander, 1997) could recognize the bovine CD18 receptor, suggesting that LKT acylation, which is necessary for activity, is not required for target cell specific binding.

Although CD11a/CD18 may be a species-specific and leukocyte-specific receptor for the two RTX leukotoxins, it may also be a receptor for binding of the non-leukocyte specific RTX general cytolysin, E. coli α-hemolysin (HlyA) to leukocytes. Lally et. al., (1997) demonstrated that leukolysis of HL60 cells by HlyA was inhibited by anti-CD11a/CD18 mAb. This finding supports earlier conclusions based on genetic

hybrid RTX toxin studies by Forestier and Welch (1990) which found that separate domains of HlyA are responsible for hemolysis and leukolysis. Therefore, the species specific domains of human and bovine leukocyte β_2-integrins may act as the species-specific receptor rendering human and bovine leukocytes susceptible to LTX and LKT, respectively. In contrast, non-leukocytic cells lack these receptors, and are thereby not susceptible to RTX leukotoxin intoxication. Furthermore, it appears that β_2-integrins on leukocytes may also act as receptors for the leukolytic activity of RTX hemolysins.

One complication to this hypothesis is that, although P. haemolytica LKT is a species-specific leukotoxin for ruminant leukocytes, this toxin exhibits low level non-species specific hemolytic activity (Murphy et al., 1995). This hemolytic activity of LKT is much less efficient than its leukolytic activity. The ability of LKT to cause hemolysis suggests that it is able to bind to erythrocytes by a mechanism independent of its species-specific, leukolytic-specific CD18 receptor. LKT binding to erythrocytes may not be mediated by a protein receptor. Instead, LKT binding to erythrocytes may involve direct interaction of LKT with membrane phospholipids. "Non-specific" binding of RTX leukotoxins to non-susceptible target cells has been observed (Sato et al., 1993; Sun et al., 1997). As suggested by the work of Forestier and Welch (1990), RTX toxin binding to erythrocytes likely involves separate domains in RTX toxins from those domains which bind leukolytic-specific CD11a/CD18 receptor domains.

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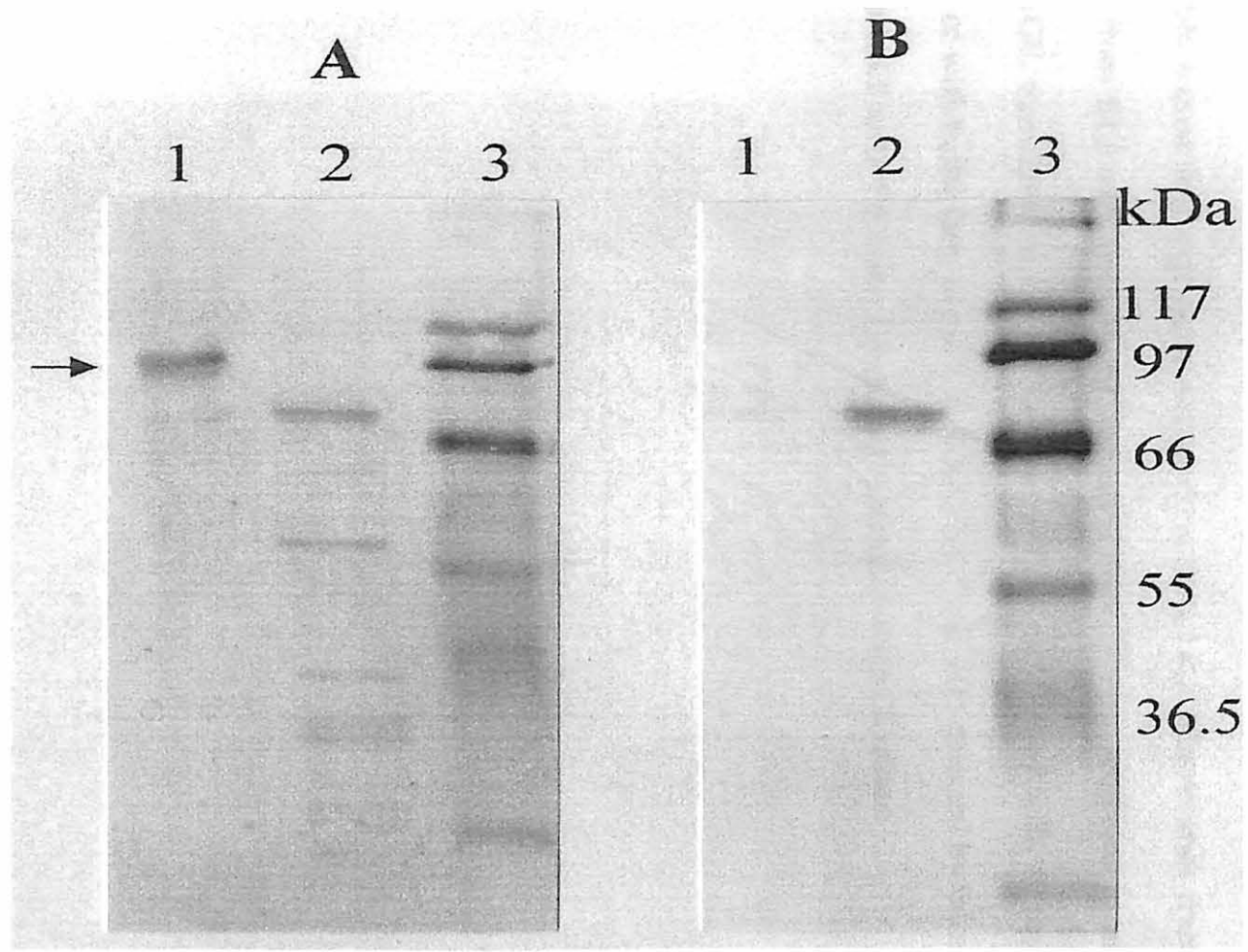
Fig. 1. Ligand blotting of LKT susceptible bovine BL3 cell and non-susceptible human Raji cell membrane proteins with LKT. Membranes fractions (16μg protein) from BL3 cells (lane 1) and Raji cells (lane 2) were subjected to non-reducing SDS-PAGE, transferred to nitrocellulose membranes and blotted with 10 μg/ml LKT in panel A or with buffer only in panel B. Bound LKT was then detected by immunoblotting with anti-LKT mAb C6. Lane 3 contained molecular weight markers.
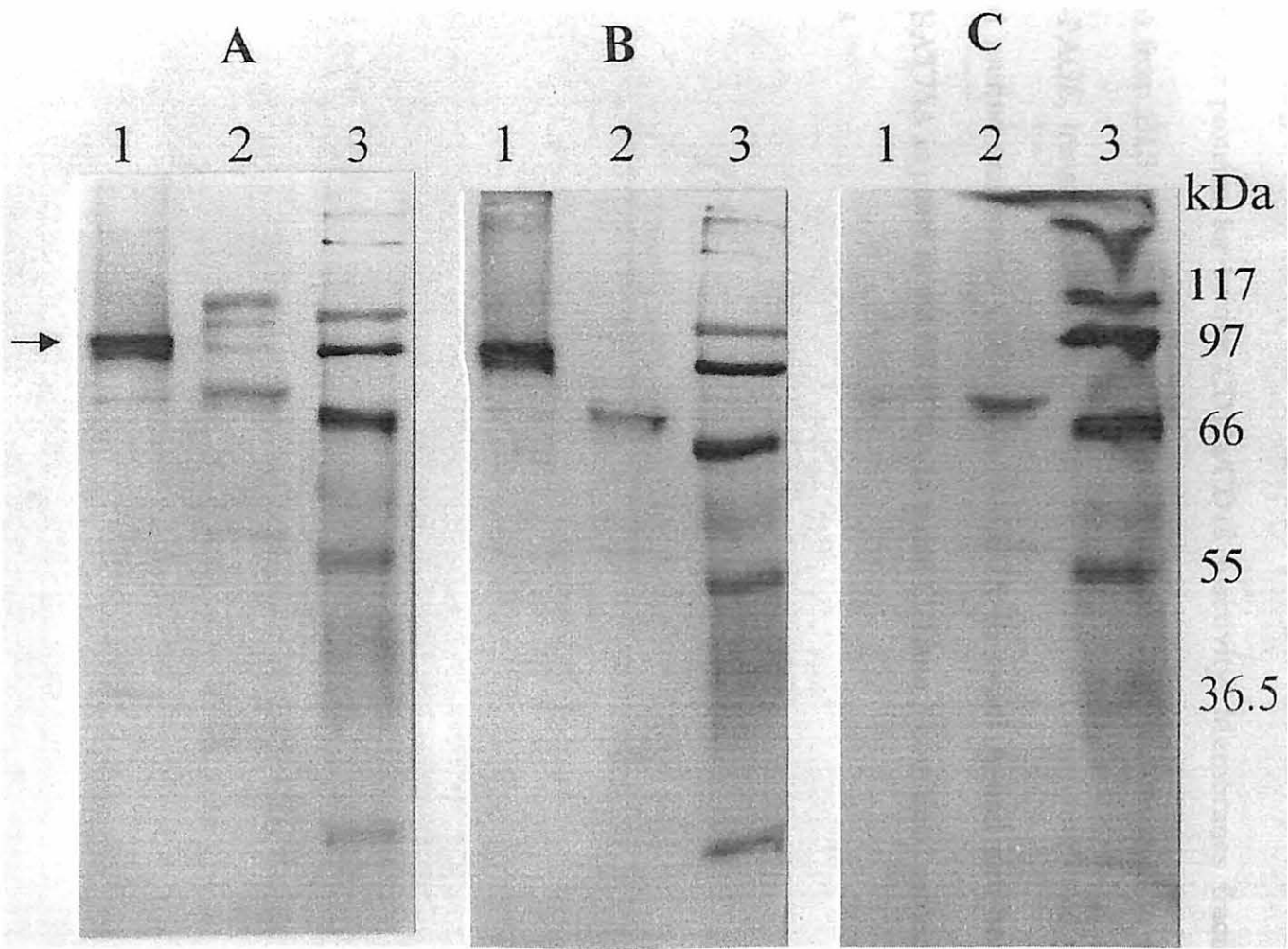
Fig. 2. Immunoblotting of LKT susceptible bovine BL3 cell and non-susceptible human Raji cell membrane proteins for bovine CD11a/CD18 reactivity. Membranes fractions (16 μg protein) from BL3 cells (lane 1) and Raji cells (lane 2) were subjected to non-reducing SDS-PAGE, transferred to nitrocellulose membranes and immunoblotted with 1:1,000 diluted murine anti-bovine CD18 mAb BAQ30A in panel A and anti-bovine CD11a/CD18 BAT75A in panel B and MUC76A in panel C. Lane 3 contained molecular weight markers.
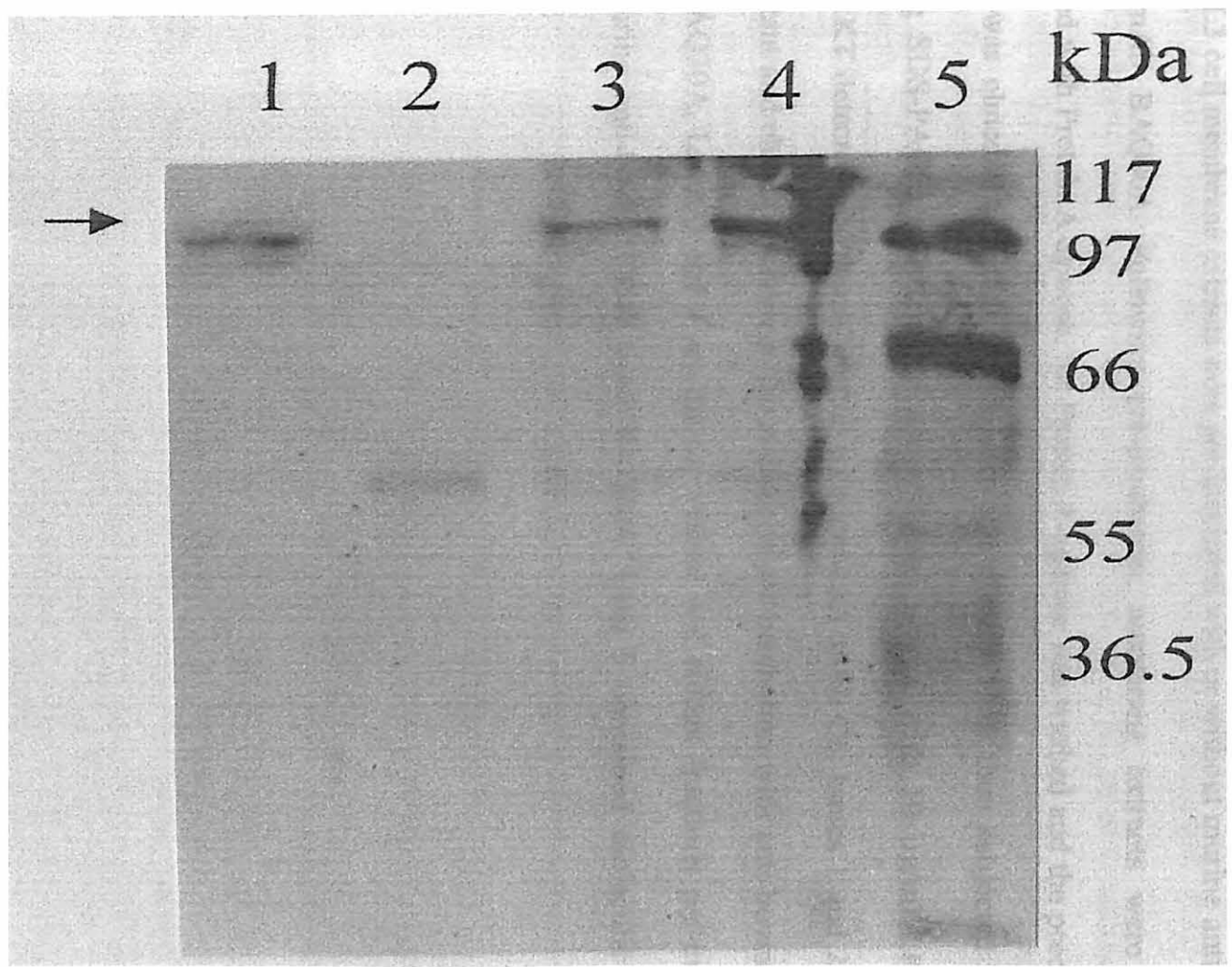
Fig. 3. Ligand blotting of isolated CD18 from LKT susceptible bovine BL3 cells with LKT. BL3 cell membrane extracts were pre-incubated with or without murine anti-bovine CD18 mAb BAQ30A. Following pre-incubation, membrane extracts were further incubated with Protein A-agarose, the Protein A-agarose was washed and the precipitated protein was eluted. The initial supernatants and elutants were then subjected to non-reducing SDS-PAGE, transferred to nitrocellulose, blotted with 10 µg/ml LKT, and bound LKT detected by immunoblotting with anti-LKT mAb C6. Lanes 1 and 2 are the supernatant and elutant fractions in the absence of pre-incubation with anti-bovine CD18 mAb BAQ30A. Lanes 3 and 4 are the supernatant and elutant fractions pre-incubated with murine anti-bovine CD18 mAb BAQ30A. Lane 5 contained molecular weight markers.

Table 1. Inhibition of LKT-induced BL3 cell leukolysis by anti-bovine β_2 integrin mAb.

BL3 cells (2×10^5) were pre-incubated with 10 µg of murine irrelevant mAb or anti-

bovine β_2 integrin mAb in 90 µl RPMI-1640 for 45 min. at 37°C, prior to addition of 10

µl containing 0.1 µg LKT and an additional 45 min. incubation. The exposed cells were

mixed with trypan blue and number of viable cells enumerated.

Treatment	% Viable cells
RPMI + murine irrelevant mAb (MOPC-21), no LKT	100 ± 14
LKT + murine irrelevant mAb (MOPC-21)	7 ± 2
LKT + anti-bovine CD18 mAb (BAQ30A)	44 ± 13
LKT + anti-bovine CD11a/CD18 mAb (BAT75A)	25 ± 8
LKT + anti-bovine CD11a/CD18 mAb (MUC76A)	6 ± 1

* Chapter VI

Lipopolysaccharide complexes with *Pasteurella haemolytica*

leukotoxin

ABSTRACT

The presence of lipopolysaccharide (LPS) in Gram-negative bacterial repeats-in-toxins (RTX) toxin preparations as well as the harsh conditions required to remove it suggests that LPS may complex with RTX toxins. Concentrated culture supernatant (CCS) preparations of the RTX toxin *Pasteurella haemolytica* leukotoxin (LKT) contained LKT and LPS as the most prominent components, with LKT and LPS constituting ≈30% and 50% of the density of the silver-stained on SDS-PAGE, respectively. CCS LKT contained 3.69 ± 0.46 mg LPS per mg protein, which was estimated to be a LPS:LKT molar ratio of ≈60:1. Subjection of the CCS LKT to preparative SDS-PAGE resulted in separation of LPS from LKT as detected by silver stained analytical SDS-PAGE, however, the LKT fraction (SDS-PAGE LKT) contained significant endotoxin activity as detected by the *Limulus* amebocyte lysate assay. Subjection of the SDS-PAGE LKT to a second preparative SDS-PAGE run resulted in reduction of the LPS:LKT molar ratio to 1:20. The target cell specificity of LKT for bovine leukocytic cells was retained by the SDS-PAGE LKT, and isolated LPS at comparable concentrations to that in CCS LKT exhibited no leukolytic activity. Addition of isolated LPS back to SDS-PAGE LKT resulted in reconstitution of a LPS•LKT complex. Immediately following reconstitution of the LPS•LKT complex, there was minimal change in leukolytic activity of the complex, but following 9.5 hrs. at temperatures from -135 to 37°C, the LPS•LKT complex exhibited increased leukolytic activity and thermal stability compared to SDS-PAGE LKT. Therefore, it appears that LPS complexes with LKT resulting in enhanced and stabilized leukolytic activity.

INTRODUCTION

Pasteurella haemolytica, the causative agent of shipping fever pneumonia in cattle, produces a leukotoxin (LKT) which is a member of the "repeats-in-toxins" (RTX) family of Gram-negative bacterial pore-forming exotoxins (8,31). The RTX family comprises a medically important group of toxins composed of two leukocyte-specific toxins, *P. haemolytica* LKT and *Actinobacillus actinomycetemcomitans* leukotoxin (LTX), as well as several hemolysins, e.g., *Escherichia coli* α-hemolysin, with broad host cell susceptibility (3,28,30). The RTX toxins share several features including mechanisms of activation, secretion, and intoxication. The genes encoding RTX toxins exhibit sequence homologies, and RTX toxins have similar structural/functional elements including putative N-terminal hydrophobic membrane-spanning domains, post-translational acylation modification sites, Ca^{2+} binding tandem nanopeptide repeats domains, and C-terminal secretion signal domains (31). Because preparations of RTX toxins also contain substantial amounts of lipopolysaccharides (LPS), the possible role of LPS in RTX toxin structure and function has been questioned (9).

LPS is a major component of Gram-negative bacterial outer membranes. The LPS molecule has amphiphilic properties and consists of a hydrophobic fatty-acyl containing lipid A, a highly charged and hydrophilic core containing 2-keto-3-deoxy-octosonic acid (KDO) substituted with phosphate and ethanolamine, and a polar, non-charged, hydrophilic repeating polysaccharide containing O-specific chain (20). LPS readily interacts with numerous biomolecules including phospholipids and membrane and serum proteins (22). Some of these interactions are non-specific and likely involving non-

saturable binding, whereas LPS binds stoichiometrically to certain proteins suggesting a specific binding process (27,34).

The relationship of LPS to RTX toxins has been controversial. The LPS content of most RTX toxin preparations have not been reported, but for those which have, the results have varied. One preparation of purified *E. coli* α-hemolysin was reported to be free of LPS based on low or non-detectable levels of KDO, lipid, and endotoxin activity, but a more highly purified preparation from the same laboratory was later reported to contain substantial amounts of the lipid A fatty acid, 3-hydroxytetradecanoic acid (4,5). The detection of significant levels of LPS in purified *E. coli* α-hemolysin lead Bohach and Snyder to conclude that LPS may be a component of the native aggregated form of α-hemolysin (4).

Evidence for a functional role of LPS in RTX toxins has also been found recently. An anti- *P. haemolytica* LKT monoclonal antibody (mAb) which blocked LKT-induced cytolysis of bovine monocytes, was not able to neutralize monokine release from these cells, suggesting that LPS in the LKT preparation was responsible for the monokine response (29). It has also been observed that LKT in cooperation with LPS may cause release of inflammatory mediators from cells which are not susceptible to LKT-induced cytoslysis (25). Czuprynski and Welch have reviewed the evidence suggesting the RTX toxins and LPS may act cooperatively and have questioned the possible functional and structural relationship of LPS and RTX toxins (9). LPS and RTX toxins are further related by participation of a transcriptional elongation factor, RfaH, which is required for maximal production of *E. coli* α-hemolysin (2,19). However, RfaH is required for both

LPS and α-hemolysin synthesis, rather than mediating LPS induction of α-hemolysin synthesis.

We have experienced difficulty in attempts to remove LPS from preparations of *P. haemolytica* LKT. We have observed that our preparations of partially purified *P. haemolytica* LKT consisted primarily of a 102 kDa LKT band and a ≈10 kDa band compatible with LPS on silver-stained SDS-PAGE (6,12). Yoo and cowokers determined that LPS could be removed from LKT by denaturation in SDS and electroporesis (35). However, dissociating agents such as 3 M guanidine, 8 M urea, or 0.25% Tween 20 diminished but not completely resolved LPS from LKT (11). Therefore, we questioned whether LPS is a contaminant of LKT preparations or rather a component of an LKT complex. We tested the hypothesis that LPS forms a structural or functional complex with LKT by comparing the stability, target cell specificity, and leukolytic activity of LPS-free LKT with a reconstituted LPS•LKT complex preparation.

MATERIAL AND METHODS

Preparation of *P. haemolytica* concentrated culture supernatant leukotoxin (CCS LKT). Concentrated culture supernatants from a wildtype *P. haemolytica* biotype A, serotype 1 strain Ok 1 (23) were prepared by inoculating 1.0 l RPMI media containing 2.2 g/l NaHCO$_3$ to an OD$_{600nm}$ = 0.25 with the organism prepared by growth overnight on 5% bovine blood agar and then in brain-heart infusion medium to late logarithmic phase. The cultures in RPMI were grown at 37°C with 70 oscillations/min. for approximately 2.5 hr. to an OD$_{600nm}$ = 0.9 to 1.0. All subsequent steps were conducted at 4°C. The bacteria were removed by centrifugation at 13,000 xg for 30 min., and culture supernatant concentrated and partially purified by fractional precipitation (0-60% saturation by addition of 361 g/l solid ammonium sulfate). Following collection of the precipitate by centrifugation at 13,000 xg for 30 min., it was resuspended at ≈0.5 mg protein/ml in 10 ml of 50 mM sodium phosphate, 100 mM sodium chloride buffer, pH 7.0 (PBS), dialyzed against the same buffer, and stored at -135°C. Samples for analytical SDS-PAGE were mixed 1:1 (v/v) with SDS-PAGE sample buffer (Sigma Chemical Co., St. Louis, MO), incubated for 3 min. in a boiling water bath, and run on either 10 or 15% gels. The gels were silver stained (Daiichi Silver Stain-II, Integrated Separation Systems, Natick, MA) (12) or transferred (Hoefer TE Semi-Dry Transfer Unit, Pharmacia Biotech, Inc., Piscataway, NJ) to nitrocellulose membranes and immunoblotted with a murine anti-LKT monoclonal antibody, C6 (6). Protein concentrations were measured using a bicinchoninic acid method (Micro BCA Protein Assay, Pierce Chemical Co., Rockford, IL).

Isolation of LPS from CCS LKT and whole *P. haemolytica* cells. A modified phenol-water procedure was used (32). For the LPS from whole cells, the wet *P. haemolytica* pellet from preparation of the CCS LKT was resuspended in 10 ml of acetone, collected by centrifugation, and dried. The dried bacterial pellet (1 g) in glass centrifuge tubes was resuspended in 10 ml of 10 mM Tris, 1 mM EDTA, pH 8.0 buffer saturated with phenol (Tris-phenol), vortexed for 30 s followed by addition of 10 ml of distilled water. For isolation of LPS from CCS LKT, 10 ml of CCS LKT were mixed with the same Tris-phenol buffer as described above. The bacterial pellet or CCS LKT mixtures were incubated at 68°C for 10 min. and vortexed 3 times, then placed on ice. Following centrifugation at 7,000 xg for 30 min. at 4°C, the water-phase was transferred to glass centrifuge tubes. For the whole cell LPS preparations, 0.1 mg RNAase (Boehringer-Mannheim Corp., Indianapolis, IN) was added and incubated at room temperature for 30 min. The water phase from each sample was mixed with 5 ml of Tris-phenol buffer and incubated at 68°C for 10 min. The mixture was vortexed 3-times during the incubation, and after centrifugation at 7,000 xg for 30 min. at 4°C, the water phase was transferred to another set of centrifuge tubes. A half-volume of chloroform was added, mixed, and after centrifugation as above, the chloroform phase was removed and discarded, and the isolated LPS in the water phase was dialyzed into 12.5 mM Tris, 96 mM glycine, 196 mM KCl , pH 8.5 buffer and stored in -135 °C. The protein and nucleic acid content of the isolated LPS was < 0.001 mg protein and <0.01 mg nucleic acid per mg KDO as measured by the Coomasie method and A_{260nm}, respectively. For the Coomasie method, 100 μl aliquots of sample were mixed with 1.0 ml Coomasie blue

reagent (0.01% Coomasie brilliant blue G250, 4.75% ethanol, 8.5% phorphoric acid) (10).

2-keto-3-deoxyoctosonic acid (KDO) assay. KDO concentrations were determined by a colorimetric microassay (17), in which 45 μl aliquots of samples were mixed with 5 μl 2N H_2SO_4, and boiled for 30 minutes in sealed microfuge tubes. Following boiling, the tubes were centrifuged at low speed to return all liquid to the bottom of the tubes, and 25 μl periodate reagent (0.04 M $NaIO_4$ in 0.125 N H_2SO_4) was added . The mixture was incubated at room temperature for 20 min., and then 25 μl arsenite reagent (2% $NaAsO_2$ in 0.5 N HCl) was added with mixing. A transient brown color developed, and after it disappeared, 50 μl 0.6% thiobarbituric acid (Sigma Chemical Co.) was added, the reaction mixture was vortexed, incubated in a boiling water bath for 15 min., and 150 μl DMSO was added immediately to each sample, followed by vortexing. After the assay tubes had cooled, the OD_{550nm} was measured. Buffer served as the blank, and a 0.1 to 6.0 μg KDO standard range (Sigma Chemical Co.) was used to quantify the KDO present in unknowns.

Endotoxin activity assay. Endotoxin activity was quantified as endotoxin units (EU) by a kinetic chromogenic *Limulus* amebocyte lysate assay (Kinetic-QCL LAL, BioWhittaker, Inc., Walkersville, MD). Assays were conducted in duplicate on 100 μl serial dilutions of samples using LAL reagent water and low-endotoxin pipet tips (USA Scientific Plastics, Inc., Ocala, FL) in flat-bottomed 96-well low-endotoxin plates (Costar, Cambridge, MA). Samples in plates were warmed to 37°C in an incubator for 10 min. prior to addition of 100 μl reagent to wells. The plates were immediately placed in a thermally controlled microplate reader (ThermoMax, Molecular Devices, Palo Alto, CA),

and the OD_{405nm} read for 15 min. at 37°C. EU was determined using a standard program for quantification of EU (SoftMax, Molecular Devices, Palo Alto, CA).

LPS dry weight determination. LPS isolated from four preparations of CCS LKT (4.55 mg protein) as described in a previous section was extensively dialyzed against water, and the dialyzate lyophilized. The lyophilized LPS was weighed on a Mettler Semi-Micro Balance (model B6, Mettler Instrument Corp., Princeton, NJ) to the nearest 0.1 mg. The amount of residual NaCl in the lyophilized LPS was determined by ion-specific electrode measurement, and the dry weight corrected for the contribution of NaCl. The KDO and EU content of the isolated, lyophilized LPS was also determined, such that the dry weight of LPS could be calculated from either the KDO or EU of various LKT preparations.

Separation of LPS from LKT by preparative SDS-PAGE. Preparative SDS-PAGE was performed on the ammonium sulfate precipitated CCS LKT which was resuspended in 1.0 ml of PBS and dialyzed against the same buffer. CCS LKT was mixed with an equal volume of SDS-PAGE sample buffer, heated in boiling water for 3 min., and then cooled on ice. Insoluble material was removed by centrifugation at 5,000 xg for 10 min. at 4°C, and the clear supernatant loaded onto the electrophoresis column of a preparative SDS-PAGE apparatus (Bio-Rad Model 491 Prep Cell, Bio-Rad Laboratories, Hercules, CA) with a 1cm 3.75% stacking gel and a 11 cm 7 % resolving gel. The electrophoresis was performed at constant current of 35 mA at 4°C for 15 hrs. Collection of fractions was begun when the dye front had migrated to approximately 3cm from the bottom of the gel column. The flow rate of elution buffer was 0.235ml/min., the A_{280nm} was recorded (Parmacia-LKB Control and Optical unit UV-1, Pharmacia Biotech, Inc)

and 4.75 ml fractions were collected (GradiFrac, Pharmacia Biotech, Inc.). SDS was removed from fractions by precipitation by adding 0.34 ml 3M KCl to fractions (the final concentration of KCl was 0.2 M) (26). Following 10 min. incubation at room temperature, the precipitated potassium dodecyl sulfate was collected by centrifuged at 700 xg for 15 min. at 4°C, the supernatant transferred to new tubes, and stored at -20°C. Protein concentration in fractions was measured on 100 µl aliquots of fractions using the Coomasie method described in a previous section, KDO was measured on 45 µl aliquots of fractions, leukotoxin activity was measured on serially diluted 25 µl aliqouts of fractions, and analytical SDS-PAGE was conducted on various fractions. The fractions containing LKT were pooled and concentrated by Centriprep 30 concentrator (Amicon, Inc., Beverly, MA). The leukolytic activity of the pooled SDS-PAGE LKT was quantified as described subsequently. To determine whether additional endotoxic activity present in the SDS-PAGE LKT could be removed, an aliquot of SDS-PAGE LKT was mixed with an equal volume of SDS-PAGE sample buffer and subjected to a second run on the preparative SDS-PAGE as described above.

Assay of leukolytic activity and quantitation of LKT activity. BL3 (CRL8037) , HL60 (CCL240), and Raji (CCL86) cells were obtained from and cultured as described by the American Type Culture Collection (Rockville, MD). Aliquots of CCS LKT, preparative SDS-PAGE fractions, or LKT samples treated in various ways were assayed for leukolytic activity by serially diluting these aliquots in wells of round-bottomed 96-well microplates containing 100 µl RPMI 1640, pH 7.2 (Sigma Chemical Co.). One-hundred µl of BL3 cell suspensions (4 x 10^6 cells/ml) was added to all wells, the plate incubated at 37 °C for 120 min. The exposure was ended by centrifugation at 700 xg for

5 min. Leukolysis was measured using leakage of the large cytoplasmic enzyme lactate dehydrogenase (LDH) into the supernatant. LDH was assayed by transfer of 100 µl of incubation supernatant to wells of a flat bottom 96-well plates, the plates were warmed to 37 °C, 100 µl of LDH assay reagent at 37 °C was added (LDL-50, Sigma Chemical Co., rehydrated by addition of 25 ml H$_2$O), and the LDH activity was measured in a thermally controlled kinetic microtiter plate reader (ThermoMax, Molecular Devices) at 340 nm for 2 min. at 37 °C. Data was reported as 10^{-3} OD/min. Maximal LDH leakage was determined by exposing cells to 0.1% Triton X100, and buffer in the place of sample aliquot was used as the background LDH leakage. The specific leakage was determined as follows:

$$\% \text{ Specific leakage LDH} = \frac{A - B}{C - B} \times 100$$

where A is the LDH leakage induced by LKT, B is the leakage in PBS, and C is the total LDH leakage induced by 0.1% Triton X100. One toxic unit (TU) is defined as the reciprocol of the sample dilution causing 50% LDH leakage from 4x10^5 BL3 cells in 200 µl at 37 °C for two hrs. Samples were tested in triplicate. For testing the target cell specificity, Raji and HL60 cells were tested for LKT or LPS induced LDH leakage as described above for BL3 cells.

Reconstitution of a putative LPS and LKT complex. LPS (370 µg) isolated from whole cells in 100 µl of 12.5 mM Tris, 96 mM glycine, 196 mM KCl, pH8.3 buffer {buffer A}) and SDS-PAGE LKT (10 µg protein in 100 µl of the same buffer) were mixed and incubated for 5 hrs. at 4°C. This mixture was loaded onto an anion-exchange

column (Mono Q HR 5/5, Pharmacia Biotech, Inc.) equalibrated in 12.5mM Tris, 96mM Glycine, 196mM KCl, pH8.3 buffer on a FPLC chromatographic system (Pharmacia Biotech, Inc.). The sample was eluted using the application buffer A and an elution buffer B composed of 0.1 N acetic acid, 2 M NaCl with a 1 hr. gradient composed of 100% buffer A for 0 to 10 min., 0-20% buffer B for 10 to 30 min., 20-50% buffer B for 30 to 48 min., and 100% buffer B for 48 to 60 min. at a flow rate of 0.5 ml/min. LPS treated identically to the reconstituted LPS•LKT complex was subjected to chromatography as described above and used as a control to locate the LPS elution peak. The A_{280nm} and conductivity were measured and 1 ml fractions were collected. Fractions containing peaks were precipitated by addition of an equal volume of 72% TCA at 4°C incubated for 30 min., the precipitate collected by centrifugation at 10,000 xg for 10 min. at 4°C, the supernatant discarded, and the pellet resuspended in 80 μl of SDS-PAGE sample buffer. LPS and LKT were detected in the precipitated fractions by analytical SDS-PAGE and silver stain and immunoblotting with anti-LKT monoclonal antibody as described in a previous section.

Immunoprecipitation of the putative LPS•LKT complex. Immunoprecipitation was conducted on 50 μl of the reconstituted LPS•LKT complex prepared as described above. The mixture was initially centrifuged at 13,000 xg for 30 min. at 4°C (all subsequent steps were conducted at 4°C) to remove any insoluble material, and then 50 μl 1:20 diluted anti-LKT monoclonal antibody C6 (4 μg) was added and incubated for 2 hr. This was followed by addition of 7 μg goat anti-murine IgG anti-sera (Sigma Chemical Co.) in a volume of 10 μl and incubation for 15 hr. The immunoprecipitate was collected by centrifugation at 13,000 xg for 30 min. and washed twice with 200 μl PBS

containing 0.03% Tween 20. The washed pellet was dissolved in 60 μl SDS-PAGE sample buffer and subjected to analytical SDS-PAGE.

Co-removal of LPS and LKT from LKT preparations by batchwise treatment with Polymyxin B-agarose. Polymyxin B-agarose (1 ml, Sigma Chemical Co.) was washed twice with and re-suspended in 0.8 ml low endotoxin water (Gibco BRL Products, Gaithersburg, MD). Equal volumes (0.8 ml) of CCS LKT, SDS-PAGE LKT or isolated LPS were mixed with washed polymyxin B-agarose at 4°C, and the polymyxin B-agarose collected by centrifugation. A 200 μl aliquot of the supernatant was removed for EU, leukolytic or protein assays conducted as described above, and the remaining 600 μl was mixed with another 0.8 ml of washed polymyxin B-agarose, and the above process repeated 2 additional times.

Comparison of the leukolytic activity and thermal stability of the reconstituted LPS•LKT complex with SDS-PAGE LKT. The reconstituted LPS•LKT complex was prepared by mixing 0.2 ml containing 30 μg LPS with 0.2 ml SDS-PAGE LKT (2.8 μg LKT), and the leukolytic activity on BL3 cells measured immediately as described in a previous section. To assess the leukolytic activity and thermal stability of LPS•LKT complex compared to the SDS-PAGE LKT following incubation at various temperature, four sets of 15 μl aliquots of SDS-PAGE LKT (15 μg protein/ml) were mixed with either 15 μl of isolated CCS LPS (3.7 μg LPS/ml) or buffer, and then one pair each of reconstituted LPS•LKT complex and SDS-PAGE LKT were incubated at −135, 4, 21, or 37°C for 570 min. Following incubation, the leukolytic activity with BL3 cells were measured by LDH leakage described in a previous section.

RESULTS

LPS content and type in CCS LKT preparations. On silver stained analytical SDS-PAGE, LKT and LPS were the most prominent components of CCS LKT preparations from *P. haemolytica* (Fig. 1A). Although the total protein and KDO content of CCS LKT varied ±50% between several consecutive preparations, LKT and LPS consistently composed about 30% and 50% of the total silver stain density of these CCS LKT preparations (data not shown). LPS isolated from the CCS LKT by phenol extraction consisted of two silver stained bands with estimated molecular mass of 10 and 17 kDa on analytical SDS-PAGE (Fig. 1B, lane 1). Likewise, LPS isolated from whole *P. haemolytica* cells was composed of two bands with similar molecular mass and banding patterns on silver stained SDS-PAGE (Fig. 1B, lane 2). The banding pattern reported herein is similar to that previously reported for LPS from *P. haemolytica* biotype A, serotype 1 (18). Therefore, it appears that the LPS in CCS LKT is similar, if not identical, to the LPS associated with the outer membrane. LPS isolated from CCS LKT (n = 4) contained 0.54 ± 0.06 % KDO (w/w) and had a ratio of $720 \pm 22 \times 10^3$ EU/mg LPS. Using a LPS monomer mass of 10 kDa , and assuming that LKT constitutes ≈60% of the CCS LKT protein (11), the molar ratio of LPS to LKT in CCS LKT would be estimated to be about 60 LPS monomers: 1 LKT monomer.

Separation of LPS from LKT. The LPS and LKT from CCS LKT were separated by preparative SDS-PAGE, as previously reported by Yoo et. al. (35). LPS eluted as two peaks in the early fractions, corresponding to its low molecular mass (Fig. 2A). A prominent protein peak eluted in the middle fractions of the run was coincident with the leukolytic activity peak following removal of free dodecyl sulfate by potassium

precipitation. This peak was determined to contain ≈20% of the protein and ≈60% of the leukolytic activity applied to the preparative SDS-PAGE.

Analytical SDS-PAGE analysis of the fractions from preparative SDS-PAGE showed that the KDO-containing fractions were composed of the 10 and 17 kDa LPS bands and a 6 kDa band not seen in the LPS isolated from CCS LKT or whole *P. haemolytica* cells (Fig. 2B, lanes 6 and 7). The origin of the 6 kDa band is uncertain, but it may be associated with the residual bromophenol dye front from the preparative SDS-PAGE. The LKT activity peak was composed of the 102 kDa LKT band and a faint 6 kDa band (Fig. 2B, lane 21). Only the 102 kDa LKT band was observed for the pooled and concentrated LKT peak from preparative SDS-PAGE (Fig. 2C, lane c). Although no LPS band was detected by analytical SDS-PAGE, significant endotoxic activity was detected by the *Limulus* amebocyte lysate assay in SDS-PAGE LKT (Table 1). Subjection of the SDS-PAGE LKT to a second run on preparative SDS-PAGE resulted in further reduction in the EU level, such that the calculated molar ratio of LPS to LKT was reduced to 1:20.

The SDS-PAGE LKT retained its target cell specificity for bovine leukocytes. The SDS-PAGE LKT was leukolytic for bovine BL3 lymphoma cells, but not for human Raji lymphoma cells or HL60 myelomonocytic leukemic cells (Table 2). Isolated LPS used at a similar concentration to that observed in CCS LKT had no leukolytic activity (data not shown).

Co-removal of LPS and LKT by polymyxin B agarose. The natural occurrence of an LPS•LKT complex was suggested by the removal of both LPS and LKT from CCS LKT preparations by polymyxin B-agarose, which binds the lipid A portion of LPS (21).

Batchwise treatment of CCS LKT with 3 successive rounds of polymyxin B-agarose resulted in complete removal of both EU and LKT leukolytic activity from CCS LKT (Table 3). EU and LKT activity were reduced at different rates by successive polymyxin B-agarose treatments, but both activities were completely removed by 3 treatments. Three rounds of polymyxin B-agarose treatment were also required to remove a similar amount of isolated LPS in the absence of added LKT (data not shown). Like CCS LKT, treatment of SDS-PAGE LKT which contained only 1:20 LPS to LKT with polymyxin B agarose resulted in removal of 98% of applied LKT activity and 93% of applied protein.

Detection of reconstituted LPS•LKT complex. Addition of isolated LPS back to SDS-PAGE LKT resulted in formation of a reconstituted LPS•LKT complex which was detected by anion-exchange chromatography. Chromatography of isolated LPS on a quaternary ammonium-type anion-exchange MonoQ HR 5/5 column developed with a non-linear 60 min., 30 ml 0-0.1 N acetic acid and 0-2.0 M NaCl gradient resulted in detection of a small breakthrough peak and a large peak eluting at 20.9 ml (Fig. 3A). Detection of LPS by analytical SDS-PAGE showed that the 20.9 ml peak contained LPS and the breakthrough peak and the inter-peak region did not (Fig. 3C, lanes 1-3). As expected, no LKT was detected as assessed by western blot (Fig. 3D, lanes 1-3). To test whether LPS could complex with LKT, 370μg LPS was mixed with 10μg LKT and incubated for 5 hrs. at 4 °C. Chromatography of this mixture on the MonoQ HR 5/5 column resulted in detection of the same breakthrough and 20.9 ml peaks as well as three additional peaks eluting in the inter-peak region (Fig. 3B). As before, the 20.9 ml peak contained LPS and the breakthrough did not (Fig. 3C, lanes 4 and 8). Peaks eluting at 13.0 and 15.1 ml consisted of both LKT (Fig 3D, lanes 6 and 7) and LPS (Fig. 3C, lanes

6 and 7). The slightly retained peak eluting at 3.0 ml did not contain either LPS or LKT (Fig. 3B and C, lane 5).

The detection of the reconstituted LPS•LKT complex was also examined using immunoprecipitation by a murine anti-LKT monoclonal antibody C6 and goat anti-murine IgG anti-sera. Following mixing of SDS-PAGE LKT and LPS as described above, immunoprecipitation of the mixture or CCS LKT preparation with anti-LKT monoclonal antibody C6 followed by goat anti-murine IgG anti-sera resulted in detection of both the LKT and the LPS bands in the washed precipitate on analytical SDS-PAGE (Fig. 4, lanes 5 and 2, respectively). The buffer control contained precipitated immunoglobulins, but no LKT or LPS (Fig. 4, lane 1). Immunoprecipitation of isolated LPS in the absence of added LKT resulted in no detected imunoprecipitated LPS (Fig. 4, lane 3). Likewise, immunoprecipitation of SDS-PAGE LKT in the absence of added isolated LPS resulted in detection of LKT, but no immunoprecipitated LPS (Fig. 4, lane 4).

Leukolytic activity and stability of reconstituted LPS•LKT complex. The effect of LPS complexation with LKT on leukolytic activity was tested immediately after complexation or following 9.5 hrs incubation at various temperatures. When the leukolytic activity of SDS-PAGE LKT was compared to the reconstituted LPS•LKT complex immediately following mixing, the leukolytic activity was similar (Table 4). The reconstituted LPS•LKT complex had enhanced leukolytic activity at -135, 4, 21 and 37°C following 9.5 hrs incubation, whereas SDS-LKT did not. The maximum enhancement was seen at 4°C. At 37°C, both the reconstituted LPS•LKT complex and

SDS-PAGE LKT were partially inactivated, but the reconstituted LPS•LKT complex appeared more thermal stable than the SDS-PAGE LKT.

DISCUSSION

Circumstantial findings suggest that LPS may play a structural and possibly a functional role in RTX toxins, but the exact nature of the relationship of LPS to RTX toxins is unclear. With respect to RTX toxin activity, it has been suspected that LPS in RTX toxin preparations may be responsible for some of the intoxication phenomena attributed to RTX toxins. Although RTX cytolytic activity appears to reside entirely with the RTX toxin proteins, particular sublytic activities attributed entirely to RTX toxins such as cytokine release may be caused by LPS rather than RTX toxins (29). Part of this uncertainty concerning the relationship of LPS to RTX toxins arises from whether LPS is a contaminant of RTX toxin preparations or whether LPS is a component of an LPS•RTX toxin structural complex (9).

Based on significant levels of the lipid A fatty acid, 3-hydroxytetradecanoic acid, in purified preparations of α-hemolysin, Bohach and Snyder proposed that *E. coli* α-hemolysin consists of an aggregated complex of α-hemolysin protein and LPS (4). Likewise, we came to consider that LPS might have a role in RTX toxin structure and function based on difficulty in removal of LPS from the RTX toxin *P. haemolytica* LKT (11). Based on these observations, we hypothesized that LPS is a structural component of LKT, rather than a contaminant.

Distinguishing between LPS as a contaminant versus LPS as a component of a complex is difficult. LPS as a contaminant assumes that LPS is unnaturally associated with LKT, but there are no specific tests for unnatural association. LPS and LKT are major components of culture supernatants (11), and both are observed in histologic sections of lung tissue from cattle naturally infected with *P. haemolytica* (33). However,

the coincidental occurrence of LPS and LKT in both culture supernatants and infected lung does not directly implicate a natural complexation of LPS with LKT. The natural occurrence of an LPS•LKT complex is supported by the co-precipitation of LPS and LKT by an anti-LKT mAb. Immunoprecipitation has been used previously to provide evidence for a natural LPS•protein complexation (15). In addition to co-precipitation of LPS and LKT by anti-LKT mAb, both LPS and LKT from CCS LKT were bound by the lipid A affinity resin, polymyxin B agarose (21), suggesting that these components may be naturally complexed. The reconstitution of an LPS•LKT complex provides further support that an LPS•LKT complex may exist naturally.

The manner in which LPS interacts with particular proteins varies from crystalline lattice forms as typified by *E. coli* major outer membrane protein O-8 where there is a 1:1 molar ratio of LPS monomers to protein monomers (34) to proteins which bind to LPS aggregates or micelles as typified by mammalian lipoprotein binding protein (LPB) or phospholipid transfer protein where the amount of LPS greatly exceed that of protein (16, 36). The high LPS content of CCS LPS•LKT complex suggests that LKT is more like the later type in which LKT is bound to aggregated LPS or LPS micelles.

The functional role of LPS in the LPS•LKT complex was not extensively examined herein. Isolated LPS at the concentrations found in CCS LKT was not cytolytic for a LKT susceptible target cell line, and the SDS-PAGE LKT retained bovine leukocyte specific leukolytic activity. Czupyrnski and collaborators reported that LPS associated with LKT may be involved in certain sublytic effects attributed to LKT (29). In some sublytic RTX effects, LPS may augment the LKT-elicited effect, whereas in others LPS may be the effector with LKT functioning as an LPS carrier. We did find that LPS may

have a structural role in stabilizing LKT activity. Like other RTX toxins, LKT has unstable activity (7), although the mechanism of this instability is not completely understood. LKT looses activity when incubated at 37 to 57°C, and this inactivation does not involve proteolysis. The reconstituted LPS•LKT complex was more stable at low to moderate temperatures than the SDS-PAGE LKT, suggesting that LPS complexation with LKT may stabilize LKT activity. The thermal instability of LKT may be associated with self-aggregation. LKT forms large aggregates with M_r of 1,000 to 8,000 kDa which are less active than LKT disaggregated to octomers or tetramers (7). It is possible that LPS may reduce LKT aggregation, thereby stabilizing LKT activity.

Preparative SDS-PAGE reduced the LPS content of LKT to <0.05 LPS monomer per LKT monomer. Although the preparative SDS-PAGE LKT did not contain detectable LPS by silver stained analytical SDS-PAGE or by KDO assay, appreciable endotoxin activity was detected by chromogenic *Limulus* amebocyte lysate (LAL) assay. The most straightforward explanation for this endotoxin activity is that the low amount of residual LPS in the SDS-PAGE LKT is the source of the endotoxin activity.

Another possible explanation for the residual endotoxin activity in the preparative SDS-PAGE LKT is that LKT itself can react with the LAL endotoxin assay. Some substances which cause false positive reactions with the LAL reagent have been characterized, but others have not (13,24). β-glycans from fungal and plant cell walls and LAL reactive cellulosic material from extracts of cuprophane dialyzers have been shown to activate LAL reagent via an alternative pathway. In one LKT preparation, reducing carbohydrate material was present in the CCS LKT preparation, and diminution of this reducing carbohydrate was associated with diminution of endotoxin activity (14). In the

present report, although KDO, the major carbohydrate component of rough LPS, was markedly reduced, endotoxin activity, although reduced, was not eliminated. It seems unlikely that significant levels of β-glycans in LKT preparations are responsible for the endotoxin activity observed. In addition to β-glycans, proteolytic enzymes can cause a true "false positive" reaction by direct degradation of the chromogenic substrate. Proteolytic activity has been detected in *P. haemolytica* culture supernatants, but this proteolytic activity can be partition from LKT (1). Therefore, the cause for the observed LAL endotoxin activity in SDS-PAGE LKT is low residual LPS associated with LKT.

ACKNOWLEDGMENTS

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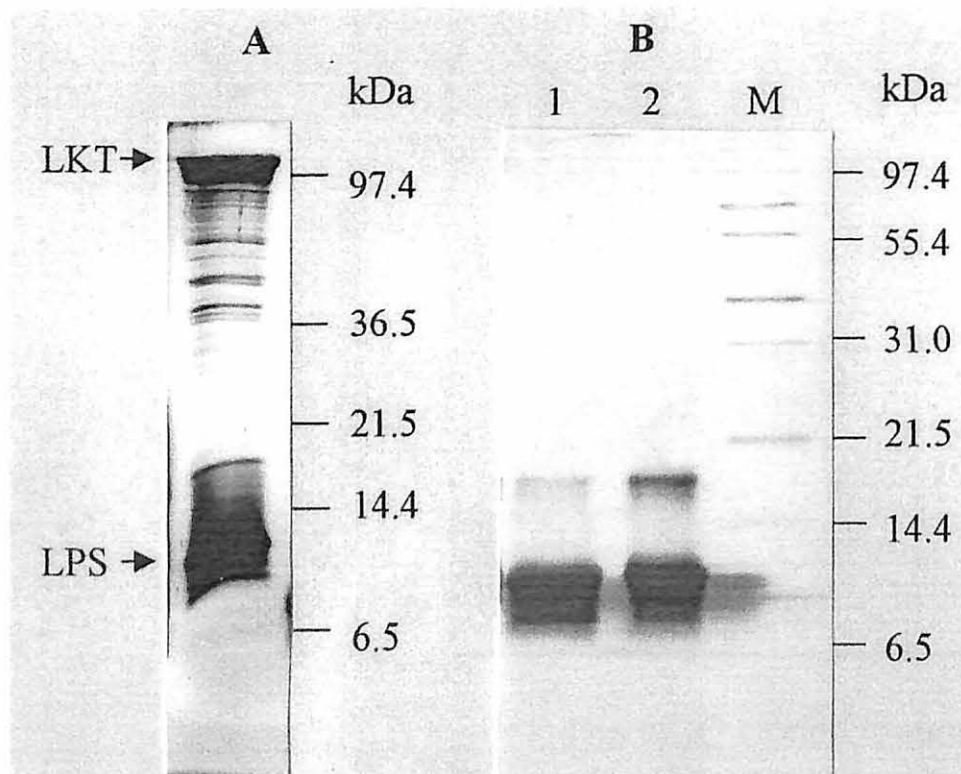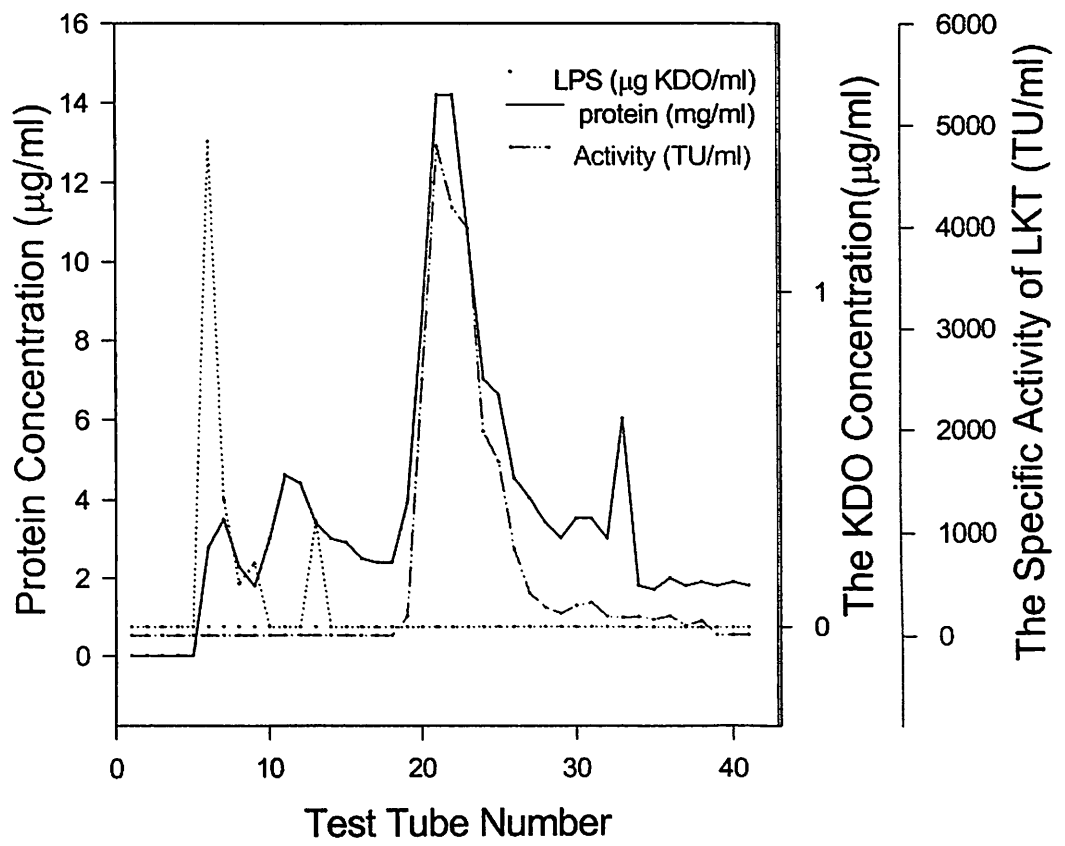
Fig. 1. Concentrated culture supernatant (CCS) LKT contained similar rough-type LPS banding pattern as that of LPS extracted from whole *P. haemolytica* cells. (A) CCS LKT (2 μg protein) and (B) phenol extracted LPS from CCS LKT (18.5 μg LPS) in lane 1 or from whole *P. haemolytica* cells (18.5 μg LPS) in lane 2 were subjected to analytical SDS-PAGE on 15% gels and silver stained. Lane M contained molecular weight markers. LKT and LPS had been previously shown to run at 97-102 and 6-14 kDa, respectively (6,11).
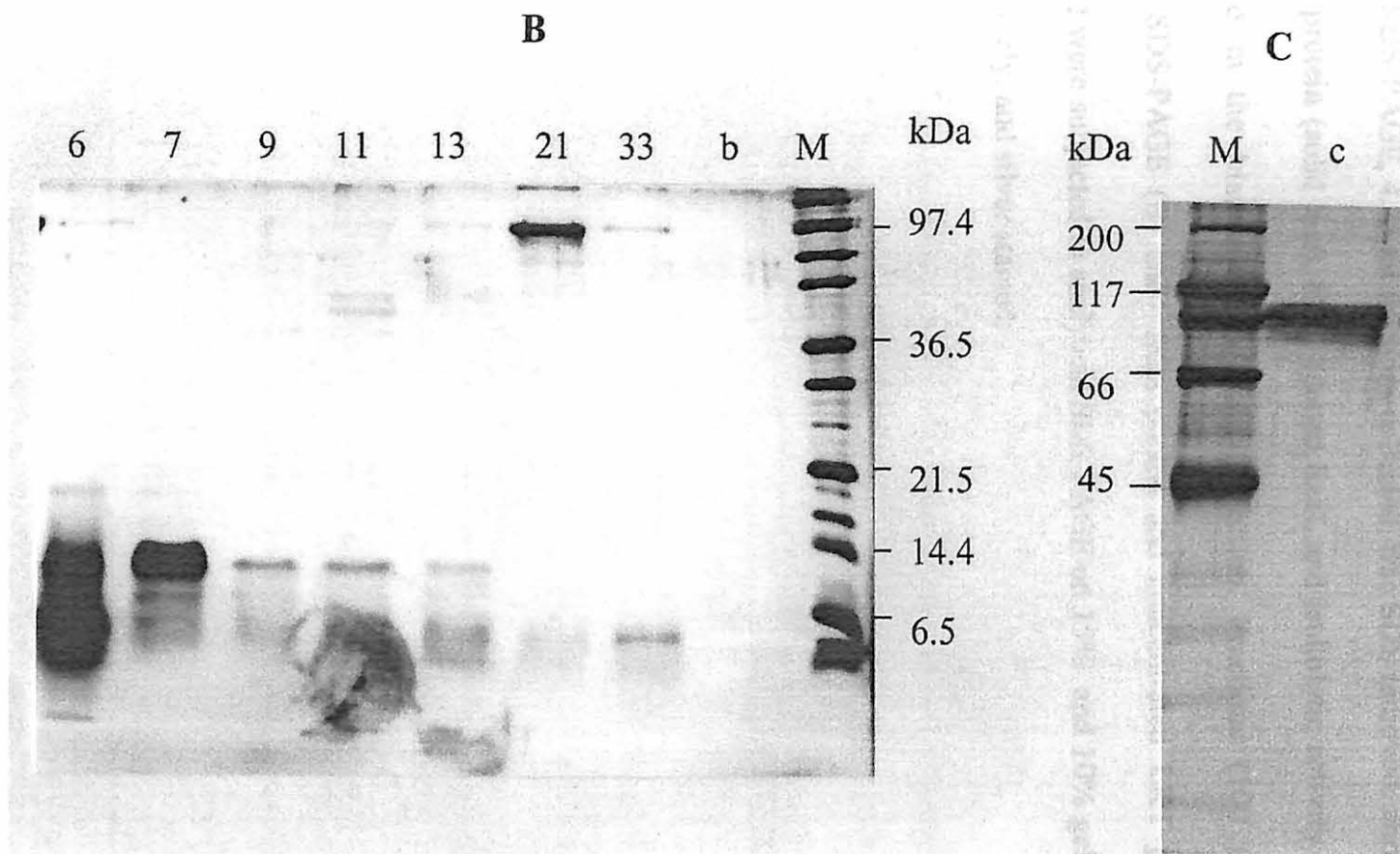
Fig. 2. Separation of LPS from LKT by preparative SDS-PAGE. (A) Concentrated culture supernatant (CCS) LKT (0.8 ml containing 2 mg protein) was subjected to preparative SDS-PAGE, 4.75 ml fractions collected, free SDS removed, and the fractions assayed for protein (solid line), KDO (dotted line), and leukolytic activity (dashed line) as described in the Materials and Methods section. Fractions (100 μl) from the preparative SDS-PAGE (B) and from pooled and concentrated LKT peak (1.0 μg protein) (C) were subjected to analytical SDS-PAGE on 15% and 10% gels for (B) and (C), respectively, and silver stained.

A
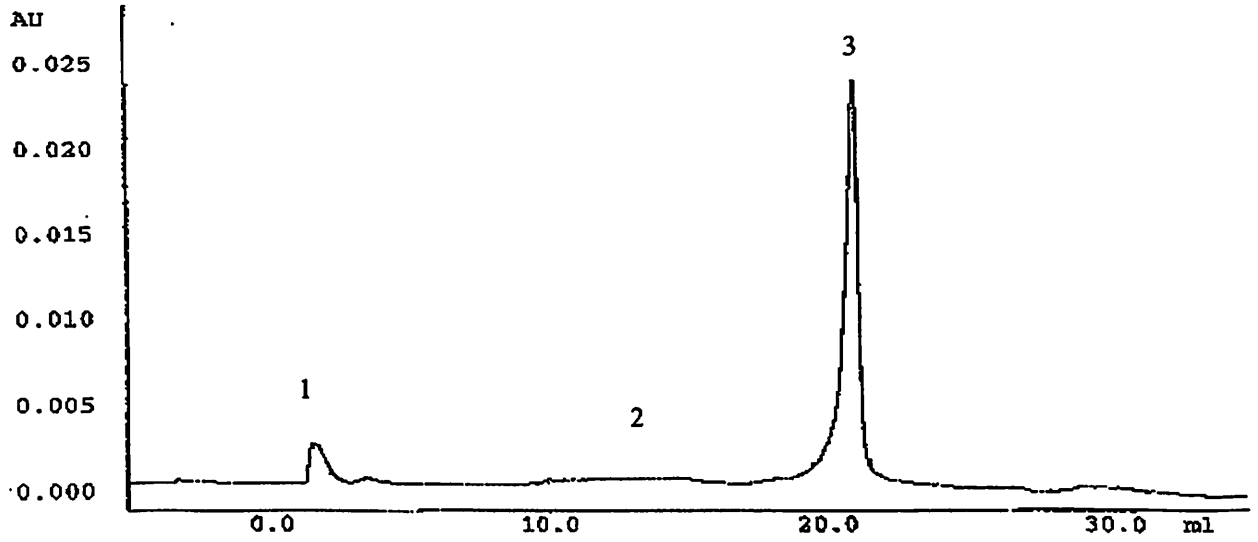
B
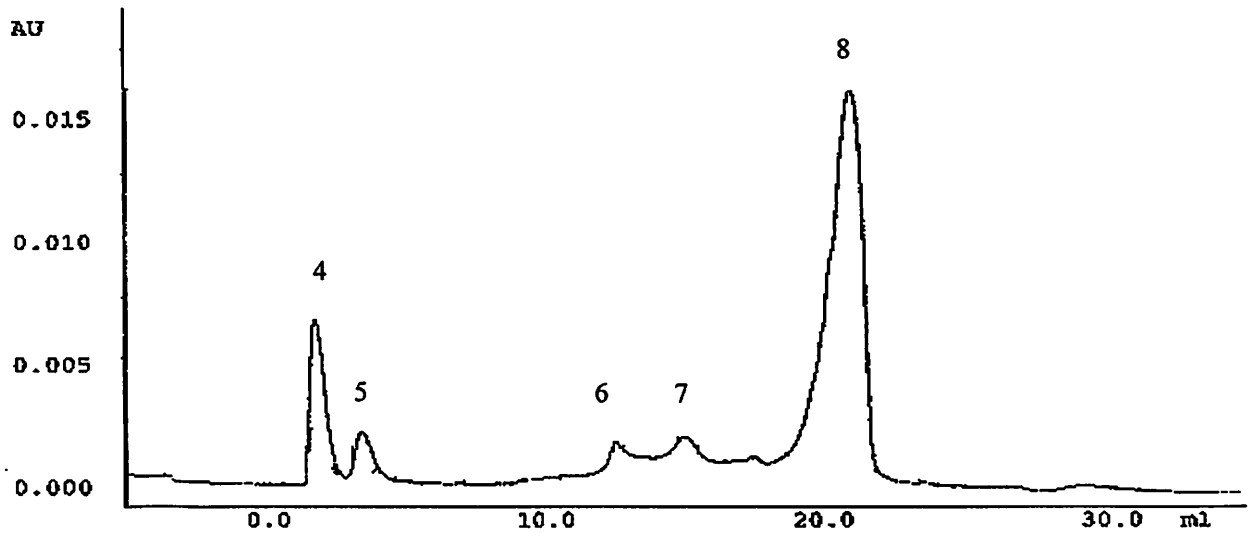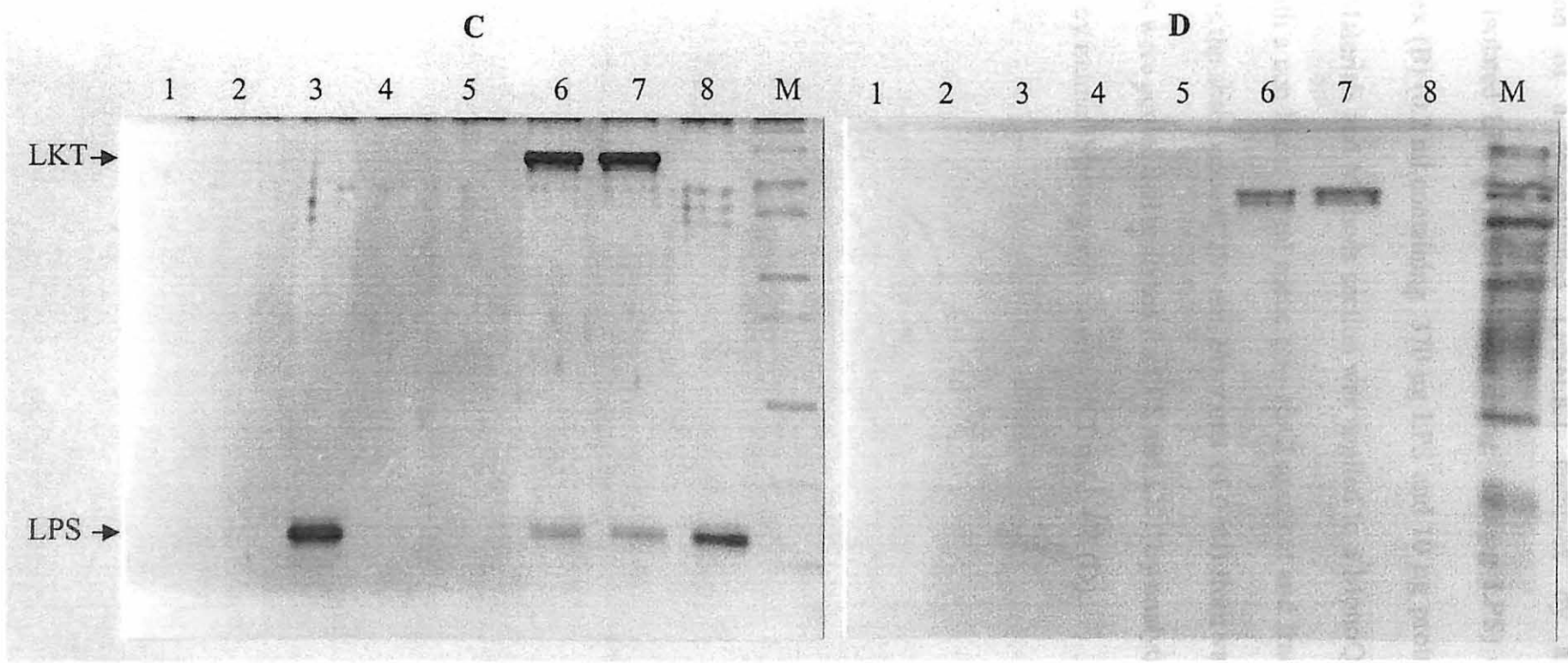
Fig. 3. Detection of a reconstituted LPS•LKT complex by anion exchange chromatography. Isolated LPS (A) (0.1 ml containing 370 μg LPS) or reconstituted LPS•LKT complex (B) (0.2 ml containing 370 μg LPS and 10 μg protein) prepared as described in the Materials and Methods section was applied to a MonoQ HR5/5 column and developed with a non-linear 30 ml acetic acid-NaCl gradient and fractions collected. The vertical axis is the absorbance at 280 nm. Fractions (1.0 ml) from various peaks and inter-peak regions were pooled and analyzed for LPS and LKT by analytical SDS-PAGE (C) and for LKT by immunoblotting with an anti-LKT mAb C6 (D).

LKT →

LPS →

8 7 6 5 4 3 2 1
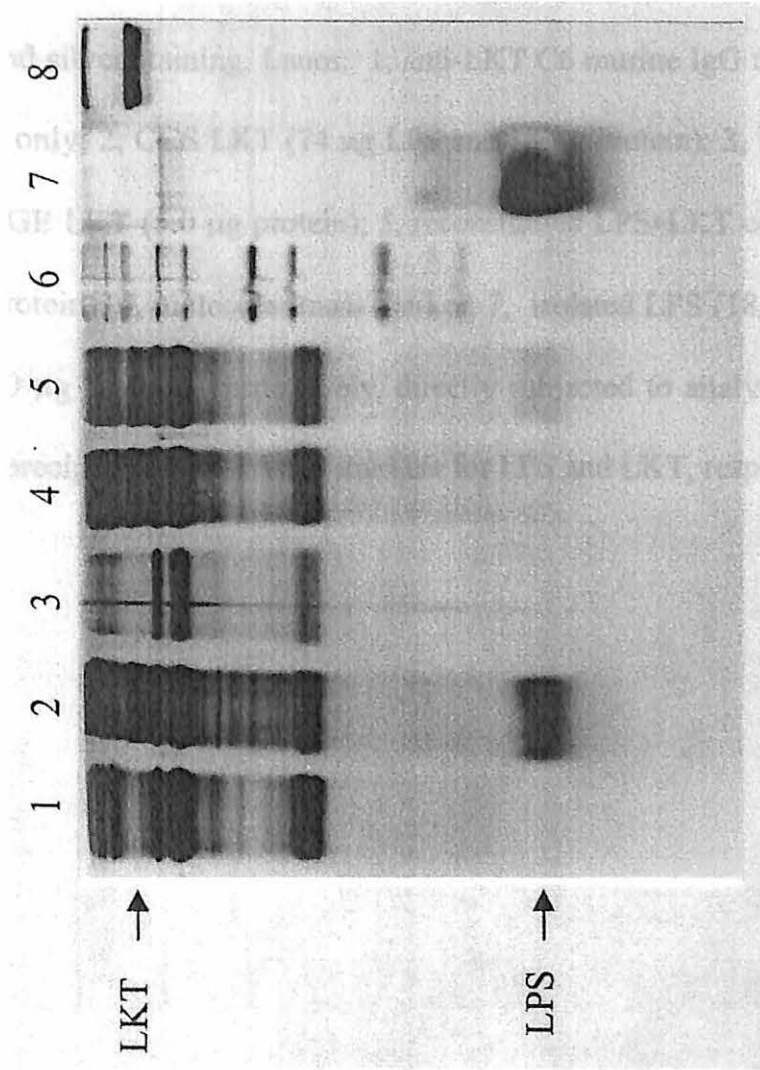
Fig. 4. Detection of a reconstituted LPS•LKT complex by co-immunoprecipitation with an anti-LKT mAb C6. Isolated LPS, SDS-PAGE LKT, or reconstituted LPS•LKT complex were incubated with anti-LKT C6 murine IgG mAb followed by precipitation with goat anti-mouse anti-sera, the precipitate washed, subjected to analytical SDS-PAGE and silver staining. Lanes: 1, anti-LKT C6 murine IgG mAb and goat anti-mouse anti-sera only; 2, CCS LKT (74 μg LPS and 13 μg protein); 3, isolated LPS (185 μg); 4, SDS-PAGE LKT (5.0 μg protein); 5, reconstituted LPS•LKT complex (185 μg LPS and 5.0 μg protein); 6, molecular mass marker; 7, isolated LPS (18.5 μg) and 8, SDS-PAGE LKT (1.0 μg protein), respectively, directly subjected to analytical SDS-PAGE without immunoprecipitation to serve as markers for LPS and LKT, respectively.

TABLE 1. LPS content of LKT preparations

LKT Preparation	LKT Specific Activity	LPS Content		
	10^3 TU/mg protein	mg LPS/mg protein	µg KDO/mg protein	10^3 EU/mg protein
CCS LKT[a]	3.88 ± 0.85	3.69 ± 0.46	22.6 ± 4.6	2,660 ± 330
SDS-PAGE LKT[b]				
1st run	125	0.063	1.38	47
2nd run	100	0.005	<0.1	3.6

[a] Values are means ± S.D. (n = 4).

[b] Values are for a single CCS preparation subjected to two successive preparative SDS-PAGE runs.

TABLE 2: SDS-PAGE LKT retains bovine leukocyte target cell specificity

LKT Preparation	LKT specific activity (10^3 TU/mg protein)[a]		
	BL3 cell	Raji cells	HL60 cells
CCS LKT	73±10	0±0	0±0
SDS-PAGE LKT	314±22	0±0	0±0

[a] Values are means ± S.D. (n = 3).

TABLE 3. Co-removal of LPS and LKT from CCS LKT by successive batchwise treatment with polymyxin B agarose

Polymyxin B-Agarose Treatment	LPS content (10^3 EU/ml)	LKT Activity (10^3 TU/ml)
Untreated	26.3	148
1X	25.0	45.0
2X	10.6	4.13
3X	0.0	0.0

TABLE 4. Complexation of LPS with LKT enhances and stabilizes LKT activity

Preparation	LKT specific activity (10^3 TU/mg protein)[a]				
	immediate following mixing	following incubated for 9.5 hrs at			
		-135°C	4°C	21°C	37°C
LPS	0±0	0±0	0±0	0±0	0±0
SDS-PAGE LKT	113±19	113±20	147±35	127±54	7±4
Reconstituted LPS•LKT complex	120±78	163±15	400±100	153±11	57±15

[a] Values are means ± S.D (n = 3).

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Chapter VII:

Pasteurella haemolytica leukotoxin and lipopolysaccharide do not act synergistically in induction of TNF-α expression by bovine lymphoma cells

Abstract

Pasteurella haemolytica leukotoxin (LKT) is complexed with lipopolysaccharide (LPS). The possibility of synergism between LPS and LKT is in question. To investigate this problem, purified *Pasteurella haemolytica* A1 LKT and LPS were used to activate bovine lymphoma cells (BL3). TNF-α mRNA in BL3 cells was quantitated. Results indicated that TNF-α mRNA level (ratio of TNF-α to β-actin mRNA) was increased more than 14-fold after incubation with sublytic LKT (0.25 TU/ml). Incubation of BL3 cells with LPS (738 EU/ml, 1 μg/ml) increased TNF-α production only approximately 2-fold. Adding LPS and LKT together induced BL3 cells to express a similar amount of TNF-α to that of LKT alone. Culture concentrated supernatant LKT, which is composed of LPS•LKT complex, also stimulated BL3 similarly to LKT alone. No synergistic effects were observed. Pre-incubating BL3 cells with antibodies to LKT receptor (CD18) reduced LKT effects from 0.109 to 0.020, but surprisingly, increased BL3 sensitivity to LPS. CD14 has been reported to be a receptor for LPS. Flow cytometry results indicated BL3 cells were CD18-positive and CD14-negative. Therefore, our results are consistent with LPS and LKT acting on target cell through different pathways. We propose that the lack of synergistic effects of sublytic LKT plus LPS might be due to the absence of CD14 on BL3 cells.

Introduction

Cattle pneumonic pasteurellosis is an economically important disease. *Pasteurella haemolytica* biotype A serotype 1 is a major etiologic agent of this disease. Lipopolysaccharides (LPS) and leukotoxin (LKT) are two important virulence factors in the pathogenesis of pneumonic pasteurellosis (Confer et al, 1995).

Stevens and Czuprynski (1995) reported that CCS-LKT induced bovine alveolar macrophages and monocytes to induce TNF-α expression. However, heat-inactivated CCS-LKT or LPS alone induced the cells to express a similar amount of TNF-α to that of CCS-LKT. Therefore, it was proposed that cytokine release might be due to the contamination of LPS in CCS-LKT. Yoo et al (1995) reported that purified LKT induces TNF-α and IL-1β expression from bovine alveolar macrophages. However, their purified LKT still contained substantial amounts of LPS (Li and Clinkenbeard, 1999).

Recently, Hsuan and his colleagues (1999) demonstrated that LKT- and LPS-induced cytokine gene expression in bovine alveolar macrophage required intracellular calcium elevation. However, cytokine expression by LPS could be distinguished from that caused by LKT using various inhibitors of increased intracellular Ca^{2+} (Hsuan et al, 1998). LKT-induced calcium elevation depends on the influx of extracellular calcium in a concentration-dependent manner, and LPS-induced intracellular calcium elevation is not dependent on the extracellular calcium (Hsuan et al, 1998). It is concluded that LKT and LPS induce cytokine gene expression through different signal pathways.

Bovine CD18 serves as the species-specific and leukocyte-specific LKT receptor (Li et al, 1999). LPS can also bind to CD18 on cell surface (Wright and Jong, 1986). Flaherty et al, (1997) demonstrated that CD11/CD18 genetic-transformed Chinese

hamster ovary (CHO) cells that were previously not sensitive to LPS became sensitive to the LPS at a dose of 100 ng/ml. Deletion of the intracellular portion of β2-integrin did not disturb the LPS sensitivity of transformed CHO (Ingalls et al, 1998), which indicates that CD18 may act as signal partners for other LPS receptors. Todd and Petty (1997) found that CD18-bound LPS was transferred to CD14 on macrophages.

CD14 on macrophages have been demonstrated to be a receptor for LPS (Wright et al, 1990). Because CD14 is anchored on the cell surface through glycosylphosphatidyl-inositol (GPI), it is believed that CD14 transfers LPS to a signal transducer on the cell surface (Ulevitch, 1995) or internalizes LPS (Vasselon et al, 1999).

Synergism among LPS, *E. coli* α-hemolysin, and other molecules has been reported. Rabbit lung pretreated with LPS became more responsive to α-hemolysin. The thromboxane release and pulmonary artery pressure in primed lung increased 15-fold more than the non-primed lung (Walmrath et al, 1994). Pre-incubation of human neutrophils with LPS enhanced the cell response to fMet-Leu-Phe, and the priming action of LPS appears to be CD14-associated (Yasui et al, 1993).

Because sublytic concentration of LKT and LPS both stimulate bovine leukocytes to express cytokines, we hypothesized that LKT and LPS could be synergistic in cytokine stimulation. Therefore, this study used reverse transcription quantitative competitive polymerase chain reaction (RT-qcPCR) (Rottman et al, 1996) to quantitate TNF-α gene expression in bovine lymphoma cells stimulated by a purified LPS and LKT. LPS-free LKT induced TNF-α gene expression. However, no synergy was observed between LPS and sublytic LKT.

Materials and Methods

Preparation of *P. haemolytica* concentrated culture supernatant (CCS) LKT.

As described before (Li and Clinkenbeard, 1999), *P. haemolytica* biotype A, serotype 1 strain Ok 1 (Panciera et al, 1984) was cultured in 1 liter RPMI-1640 (Sigma Inc. St. Louis, MO) at 37^0C to logarithmic phase. The following steps were performed at 4^0C. After removing bacteria by centrifugation (13,000 xg for 30 min), solid ammonium sulfate was added into the supernatant to 60% saturation with slow agitation. The precipitated proteins were collected by centrifugation (13,000 xg for 30 min), and the pellet was dissolved and dialyzed against phosphate buffered saline (PBS, 100 mM sodium chloride, 50 mM sodium phosphate, pH 7.0) overnight.

Isolation of LPS from *P. haemolytica*. *P. haemolytica* LPS was isolated from CCS LKT as previously decribed by the modified phenol-water procedures (Li and Clinkenbeard, 1999). Purified LPS was dialyzed against distilled water for two days with six changes and lyophilized. For these studies, lyophilized LPS was reconstituted at 2mg/ml.

Purification of LKT. Preparative SDS-PAGE was used to purify LKT (Li and Clinkenbeard, 1999). Briefly, 1ml (2mg protein/ml) CCS-LKT was mixed with 1ml of 2x SDS-sample buffer and boiled for 3 minutes. Insoluble material was removed by centrifugation (10,000 xg for 5 min). The clear supernatant was loaded onto the preparative SDS-PAGE gel column (10 cm 7% resolving gel with 1 cm 3.75% stacking gel). Electrophoresis was performed with constant electric current (35 mA) at 4^0C over night. The LKT peak was collected, concentrated by centriprep-30 (Amicon Inc., Beverly, MA), and subjected a second time to preparative SDS-PAGE as described

above. LKT peak was collected and concentrated. Three molar KCl was added to the fraction to a concentration of 0.2 M and incubated for 10 minutes at room temperature. The potassium dodecyl sulfate was removed by centrifugation (1,000 xg for 10 minute). The supernatant (purified LKT) was stored in −135 ^{0}C. The purified LKT is subsequently referred to as SDS-PAGE LKT.

Determination of optimal LKT sublytic level for BL3 activation. BL3 cells (5×10^6 in 5 ml) were incubated in RPMI-1640 with 0, 0.125, 0.25, 0.5, 1, 2 TU/ml of purified LKT. After incubation at 37 ^{0}C for two hours, supernatants were separated from cells by centrifugation at 700 xg for 15 minutes, and LDH determined. Similar concentration of LKT were also mixed with BL3 cells in MEM medium containing 10% FBS and incubated at 37^{0}C for two hours. Cells were collected by centrifugation at 700 xg for 15 minutes and RNA extracted and quantified.

Measurement of LKT lytic activity and endotoxin activity. As previously described (Li and Clinkenbeard, 1999), LKT lytic activity was quantitated according to LDH leakage, which is quantitated by spectrophotometically using LDL-50 reagent (Sigma Co., St. Louis, MO). The endotoxin activity was measured by the kinetic quantitative chromogenic LAL kit (Biowhittaker, Maryland) as reported previously (Li & Clinkenbeard, 1999).

BL3 cells activation. BL3 cells were suspended in MEM medium containing 10% FBS at 10^6 cells/ml. Five ml of BL3 cells were loaded into each well of 6-well plates. Each well had 12.5 µl of RPMI-1640 buffer containing 100 TU LKT, or 3688 EU LPS, or 100 TU LKT plus 3688 EU LPS, or CCS-LKT (100 TU LKT with 3688 EU

LPS), or RPMI-1640 alone. Plates were incubated at 37^0C for two hours before extraction of RNA.

RNA extraction: Following incubation, BL3 cells in wells were pooled into 15ml sterile plastic centrifuge tubes. After centrifugation at 700 xg for 5 min., the supernatants were removed, pellets were vortexed for 1 min, and 0.5 ml of RNAstat-60 (Tel Test B, Friendswood, TX) was added to the pellets. The cells were lysed by pipetting up and down ten times. The lysed cells were transferred to 1.5 ml microcentrifuge tubes, chloroform (0.1 ml) was added to each tube and tubes were vortexed for 30 seconds. After centrifugation at 11,000 xg for 15 min., the upper layer liquids was transferred to new 1.5 ml microcentrifuge tubes. Equal volumes of isopropenol and 1/10 volume of 3 M sodium acetate were added to each tube and mixed. Tubes were stored at -20^0C for 1 hour and precipitated RNA was collected by centrifugation at 11,000 xg for 15 minutes. The pellets were washed with cold 70% ethanol once and air-dried. Each pellet was dissolved in 50 µl DEPC (diethyl phosphoryl cyanide) treated water, and RNA concentrations were measured by OD260 (1OD = 50mg RNA/ml). The RNA preparations were stored at -20^0C for use.

Reverse transcription of mRNA: Six µg RNA samples in 60µl DEPC-treated water were mixed with 12 µl oligo-dT15 (25pmol/µl), heated to 72^0C for 7 minutes, and chilled on ice. To each sample, 30µl 5X RT buffer, 15 µl 0.1 M DTT, 24 µl dNTPs (2.5 mM each), 3 µl RNAsin (40 U/ml), 3 µl Superscript Reverse Transcriptase (100U/ml, GIBCO BRL), and 3 µl DEPC-water were added. The samples were incubated at 45^0C for 1 hour, 94^0C for 10 minutes, and immediately chilled on ice.

Polymerase chain reaction: As previously described (Rottman et al, 1996), a 600-base pair competitive DNA fragment was obtained by overlapping extension PCR (Ho et al, 1989). The competitive fragment was diluted with DEPC-treated water to 500 pg/µl and then 5-fold serially diluted 11 times to obtain 12 concentration of competitor solutions. The bovine-specific primer sequences for β-actin and TNF-α are shown in TABLE 1. The cDNA samples were 4-fold diluted with DEPC-treated water. The template cDNA for amplification of β-actin was diluted 1000-fold, and the template cDNA to amplify TNF-α was diluted 10-fold. For each series of 12 reactions, 141.4 µl water, 32.5 µl 10x PCR buffer, 19.5 µl 25mM $MgCl_2$, 26 µl dNTP (2.5mM each, Takara Shuzo Co., LTD, Japan), 32.5 µl diluted cDNA, 6.5 µl primer mixture, and 1.6 µl Taq polymerase (5 U/ml, Takara Shuzo Co. LTD, Japan) were mixed together (master solution). In each well of 96-well thermquick PCR plate (Greiner Labortechnik Ltd. Stonehouse, Glos., U.K.), 5 µl of competitive fragment solution and 20 µl of master solution were deposited. One drop of mineral oil was used to cover each well. The plate was put on thermocycler (PIC-100, MJ Research, Inc., Waltham, MA), and program set as 94^0C for 5 min., then cycled at 94^0C for 30 seconds, 60^0C for 45 seconds, and 72^0C for 60 seconds. The cycles continued for 40 times. Finally, the samples were incubated at 72^0C for 5 min.

Quantification of RT-qcPCR products. As reported by Rottman et al (1996), PCR products were subjected to electrophoresis for one hour in 2% agarose gel. Gels were stained with ethidium bromide and photographed under UV light. The photograph was scanned into digital files, and the digital images were analyzed by NIH Image software (Scion Corp, Frederick, Maryland). The fluorescence of the target and

competitor bands were measured in each lane and expressed as area under curve. The mRNA ratio of TNF-α and β-actin were calculated as described previously (Rottman et al, 1996).

Detection of CD14 and CD18 on bovine lymphoma cells. One ml of BL3 cells $(2 \times 10^6$ cells/ml) was collected, washed twice with PBS, and suspended in 1 ml 2μg/ml primary murine anti-bovine CD14 (M9, VMRD) or anti-bovine CD18 (BAQ30A, VMRD Inc., Pullman, WA) antibodies in PBSA buffer (PBS containing 0.1% sodium azide). After incubation on ice for one hour, the cells were collected at centrifugation of 7,000 xg for 5 minutes and washed twice with PBSA buffer. The cells were then re-suspended in 1ml of 0.8 μg/ml goat anti-mouse FITC conjugated antibody (Caltag Co.) in PBSA buffer and incubated in the dark on ice for 1 hour. The cells were collected by centrifugation (7,000 xg for 5 min), washed with PBSA twice, re-suspended in 1 ml PBSA, and subjected to flow cytometric analysis (FACStarPLUS, Becton-Dickinson Immunocytometry Systems, Maintain View, CA). Ten thousand cells were counted. BL3 cells treated with PBSA buffer instead of primary antibody solution were used as negative control.

Results

Quality of purified LPS and LKT. As TABLE 2 shown, CCS-LKT at a protein concentration of 1 mg/ml had lytic activity of 4600 TU/ml, and endotoxin activity of 1.36×10^7 EU/ml. Purified LPS at a concentration of 2mg/ml had specific endotoxic activity of 7.4×10^5 EU/mg. Purified LKT (SDS-PAGE LKT) dissolved in Tris-buffer (12.5 mM Tris, 96 mM glycine, 196 mM KCl, pH8.3) at a concentration of 0.54 mg/ml had. lytic activity of 93,000 TU/ml, and endotoxin activity of 2233 EU/ml.

Determination of optimal LKT sublytic level for BL3 activation. As Fig. 1 shown, at high concentration of LKT, most cells were lysed. Therefore, the RNA yield was proportionally low. At the activity level of 0.25 TU/ml LKT, approximately 15% LDH was released from the cells, and the RNA yield was over 95%. However, at the 0.5 TU/ml LKT activity level, greater than 30% LDH was released, and the RNA yield was less than 60%. Therefore, in subsequent experiments, 0.25 TU/ml LKT was used as the optimal sublytic activity level to induce cytokine gene expression.

SDS-PAGE LKT induced TNF-α expression in BL3 cells. BL3 cells (10^6 cells/ml) were incubated with 0.25 TU/ml SDS-PAGE LKT at 37 ^{0}C for two hours. The total RNA from the activated BL3 cells was extracted, and RT-qcPCR was performed. The amplified products were quantitated (Fig. 2). SDS-PAGE LKT treatment induced TNF-α expression level in BL3 cells 14-fold compared to negative controls (TABLE 3).

Anti-bovine CD18 antibodies inhibited LKT effects. In order to identify whether LKT-induced TNF-α expression was CD18-associated, a bovine specific anti-CD18 antibody BAQ30A (10 μg/ml) was pre-incubated with 5 ml BL3 cells (10^6

cells/ml) in MEM medium containing 10% FBS at 37⁰C for one hour. Purified LKT was added at an activity level of 0.25 TU/ml and incubated at 37 ⁰C for two hours. After RNA extraction, followed by RT-qcPCR, the results indicated that TNF-α gene expression was reduced by > 4-fold compare to LKT alone (TABLE 3). Therefore, CD18 was proposed to mediate LKT-induced TNF-α expression.

LPS induced TNF-α expression in BL3 cells. BL3 cells (5 x 10^6 in 5 ml) were incubated with 1.02μg/ml LPS, which corresponded to the amount of LPS in CCS LKT at an activity level of 0.25 TU/ml. The mRNA was extracted, and RT-qcPCR was performed. The ratio of TNF-α to β-actin mRNA was 0.005 at negative control. After incubation with LPS in the medium, the TNF-α expression slightly increased to 0.010. The irrelevant antibodies MOPC21 did not affect LPS action, the LPS induced TNF-α expression level was twice of that of the negative control (TABLE 3). Surprisingly, in the presence of anti-CD18 antibodies of BAQ 30 A, TNF-α expression increased from 0.005 for the negative control to 0.037 with LPS induction (TABLE 3).

Lack of LKT and LPS synergistic effect on the TNF-α expression in BL3 cells. In the presence or absence of MOPC21 antibodies, BL3 cells (5 x 10^6 in 5 ml) were incubated with SDS-PAGE LKT, LPS plus LKT, or CCS LKT, and an extracted RNA was used for RT-qcPCR. For the negative control, ratio of TNF-α to β-actin mRNA was the same as previous result, 0.005. The LKT plus LPS induced mRNA ratio of TNF-α to β-actin to 0.064. The CCS LKT induced the ratio to 0.080. There was significant difference between negative control and experimental group of LKT plus LPS or CCS LKT. However, compare to the LKT alone induced level, 0.085, there was no difference among them (TABLE 3).

CD18 and CD14 on BL3 cells. To determine why BL3 cells responded differently to LPS and to LKT, flow cytometry was performed to determine the presence of CD14 and CD18 on BL3 cells. Results indicated that >99% of BL3 cells were CD18 positive (Fig 3C). Conversely, <1% of BL3 cells were CD14 positive (Fig. 3B). Therefore, BL3 cells are CD18-positive and CD14-negative cells.

Discussion

Cytokine release is an important event in pathogenesis of bovine pneumonic pasteurellosis. Due to the difficulty of purification of LKT, the question of whether the increase of cytokine expression with LKT is from LPS contamination has not been resolved (Stevens and Czuprynski, 1995). We used preparative SDS-PAGE to obtain the purified LKT (SDS-PAGE LKT) which was substantially free of LPS (0.02 EU/ TU), and used it to stimulate the BL3 cells at a sublytic level. It was found that SDS-PAGE LKT stimulated 14-fold increase in TNF-α expression, whereas LPS whose amount corresponded to that in CCS-LKT resulted in only 2-fold TNF-α expression increase in the BL3 cells. LPS induces a high level of TNF-α expression in macrophages (Steven and Czuprynski, 1995). Therefore, the difference in LPS induced TNF-α expression in BL3 cells might be due to the different expression level of CD14.

It has been found that CD18 is receptor for LKT (Li et al, 1999). As expected, anti-CD18 antibodies blocked LKT stimulation. Although CD14 is a receptor for LPS (Wright et al, 1990), evidence also indicate that LPS binds to CD18 (Wright and Jong, 1986), and is then transferred to CD14 (Todd and Petty, 1997). In our experiments, we observed that LPS induced TNF-α expression from BL3 cells. LPS-stimulated TNF-α expression from bovine macrophage has been reported (Yoo et al, 1995). Flow cytometry indicated that there is no detectable CD14 expression on the BL3 surface and significant amount of CD18 on BL3 cells. Therefore, we propose that surface exposed CD14 might be essential for LPS-sensitivity.

Although both LKT and LPS bind to CD18, no obvious competitive inhibition was observed in our experiment. It is interesting to note that anti-CD18 antibodies

enhanced BL3 cell sensitivity to LPS. That LPS binding protein facilitates LPS binding to LPS receptor on cell surface has been reported (Hailman et al, 1994). Anti-CD18 antibodies might facilitate LPS binding to CD18 by changing CD18 conformation or transferring LPS to CD18, which increased BL3 sensitivity to LPS.

Previous reports of synergism between LPS and RTX toxins have appeared. Walmrath et al (1994) reported that pre-treatment of rabbit lung with LPS enhanced the response to hemolysin. The synergistic action of LPS is reported through cell-surface attached CD14. Our results indicated that LPS had no synergistic effects on sublytic LKT in vitro in BL3 cells. Therefore, we propose that the synergistic effects might be through different receptors. BL3 cells do not have CD14, therefore, no synergism between LKT and LPS occurs.

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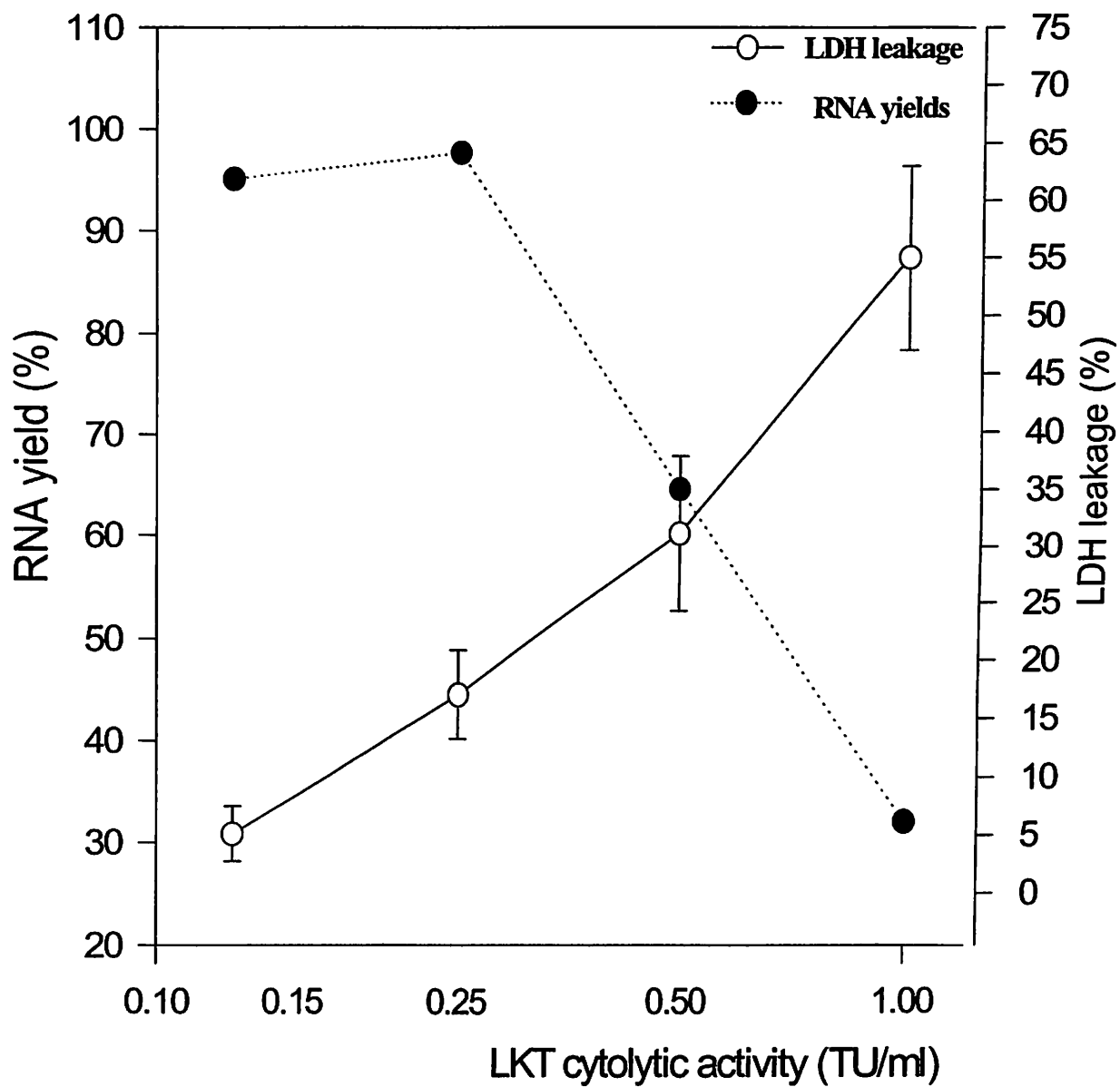# LDH leakage and RNA yields depend on LKT lytic activity

Fig. 1. Relations among LKT activity, LDH leakage, and RNA yield.

LKT (0, 0.125, 0.25, 0.5, 1 TU/ml) were incubated with 5 ml BL3 cells (10^6 cells/ml) in

RPMI-1640 or MEM medium with 10% FBS at 37^0C for two hours. After centrifugation,

the supernatants were used to determined LDH leakage by LDL-50 reagent, and the cells

were extracted for RNA, respectively. The amount of RNA was determined by OD260.

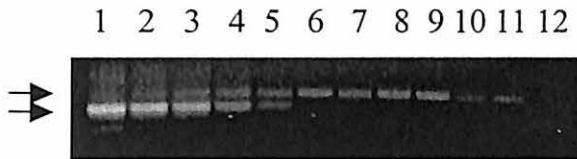RNA yield was obtained by normalizing to RNA production with 0 TU/ml LKT.
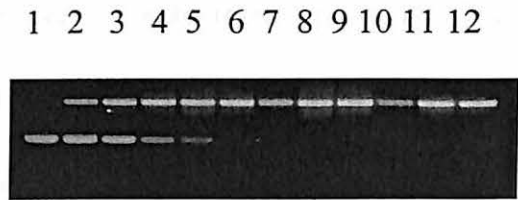
Fig. 2 RT-qcPCR products of β-actin (A,C) and TNF-α (B, D)

BL3 cells (5x10^6 in 5ml MEM plus 10% FBS medium) were incubated with 12.5 μl RPMI-1640 buffer (A, B) or 12.5 μl 100 TU SDS-PAGE LKT (C, D) at 37 ^{0}C for two hours. Extracted mRNA (6μg) of each sample were reverse transcripted to cDNA in 150μl buffer. The cDNA were diluted 4,000 fold for PCR to quantitate β-actin, or 40-fold for PCR to quantitate TNF-α. 2.7 μl of cDNA was added to each well for qcPCR. 3.7x10^8 competitive fragment molecules were added into well 1. From well 1 to 12, the amount of competitive fragment decreased 5-fold serially. After PCR, the products were loaded on 2% agarose for separation by electrophoresis. For each panel, the upper bands are target production, and lower bands are competitor production.
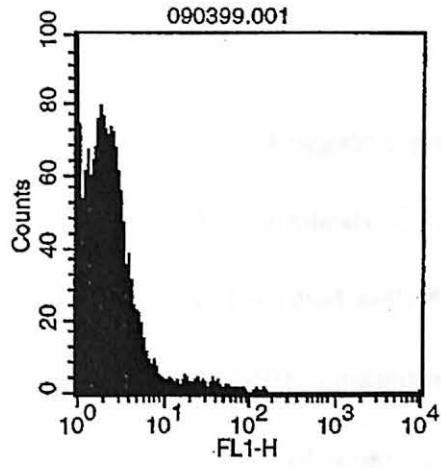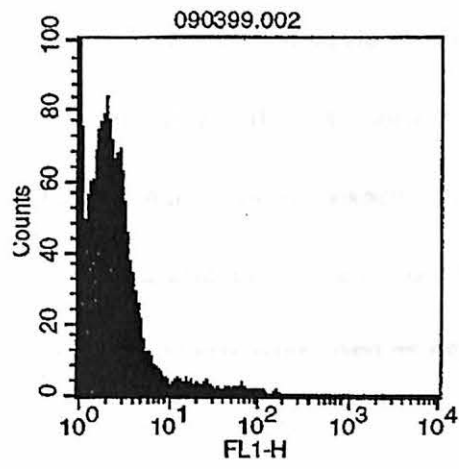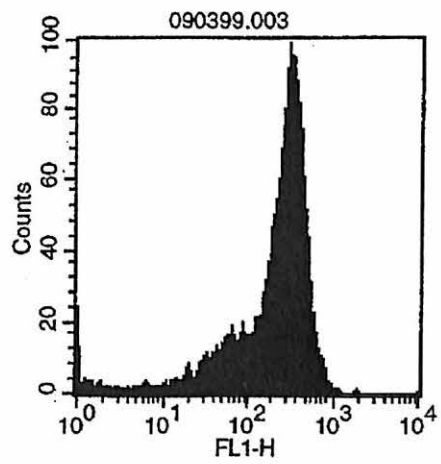
Fig. 3 BL3 cells are CD18-positive and CD14-negative proved by flow cytometry.

A: Negative control. B: anti-CD14 antibody. C: anti-CD18 antibody.

BL3 cells (2×10^6 cells) were collected and washed with PBS twice, and suspend in 1 ml 2μg/ml primary antibodies PBSA buffer (PBS containing 0.1% sodium azide). After incubation on ice for one hour, the cells were collected by centrifugation of 7,000 xg for 5 minutes and washed with PBSA buffer twice. The cells were then re-suspended in 1ml 0.8 μg/ml goat anti-mouse antibody FITC conjugate (Caltag Co.) in PBSA buffer, and incubated in dark on ice for an hour. The cells were collected by centrifugation (7,000 xg for 5 min) and washed with PBSA twice, and re-suspend in 1 ml PBSA, and subjected to flow cytometric analysis. Ten thousand cells were counted. PBSA buffer instead of primary antibody solution treated BL3 cells were used as negative control.

TABLE 1. Primer sequences for bovine β-actin and TNF-α

Target	Primer A	Primer B
β-actin	5'ACCAACTGGGACGACATGGAG-3'	5'GCATTTGCGGTGGACAATGGA-3'
TNF-α	5'CTGTGGGCTTTTGGGTTTTTCTG-3'	5'CTCTTCCGCTTTCTGAGGTTAGA-3'

TABLE 2: Content of LPS and LKT in preparations

Preparations	Protein (mg/ml)	LPS (mg/ml)	Cytolytic Activity (TU/ml)	Endotoxin (EU/ml)
CCS-LKT	1.0	18.9*	4,600	1.36×10^7
SDS-PAGE LKT	0.54	0.003*	93,000	2,233
LPS	0	2.0	0	1.44×10^6

* Calculated from the endotoxin activity/ LPS specific endotoxin unit

TABLE 3: The amount of LKT, LPS in each inducing reagents, and the induced TNF-α/β-actin mRNA ratio

Inducing reagents	LKT activity (TU/ml)	protein (µg/ml)	Endotoxin (EU/ml)	LPS (µg/ml)	mRNA ratio TNF-α/β-actin (in buffer)	mRNA ratio TNF-α/β-actin (with MOPC 21 MAb)	mRNA ratio TNF-α/ β-actin (with BAQ 30A MAb)
Negative control	0	0	<0.01	<10^{-5}	0.006	0.005	0.007
CCS-LKT	0.25	5.4×10^{-2}	737	1.02	0.080	0.099	0.046
SDS-PAGE LKT	0.25	1.8×10^{-3}	<0.01 (7.6×10^{-3})	<10^{-5} (8×10^{-9})	0.085	0.109	0.020
LPS	0	0	737	1.02	0.010	0.009	0.037
LPS+LKT	0.25	1.8×10^{-3}	737	1.02	0.064	0.086	0.043

Conclusion

LPS is a major component in LKT preparations, and it plays some roles in stabilizing LKT structure and increasing LKT activity by forming a complex. Removal of LPS from LKT does not affect LKT function and target cell specificity. LKT cell specificity is mediated by bovine CD18. Though human CD18 and bovine CD18 are highly homologous, LKT recognizes bovine CD18 but not human CD18. Through CD18, LKT mediates several species-specific actions, which include leukolysis and induction of cytokine expression. In sublytic level, LKT stimulates TNF-α expression from BL3 cells significantly. However, LPS is not very effective in inducing TNF-α expression from BL3 cells. The difference might be caused by the absence of CD14 expression on BL3 cells. As expected, pre-incubation of BL3 cells with anti-CD18 antibodies inhibited LKT action of leukolysis and induction of TNF-α expression. Although LKT complexes with LPS, no synergism is observed between sublytic LKT and LPS in inducing cytokines expression by BL3 cells.

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