CHAPTER II

LITERATURE REVIEWS

Types of bacteria

Bacterial cells have a wide variety of sizes and shapes. They are generally 0.2 to 2 μm in diameter and 1 to 6 μm in length (Koneman et. al., 1997). Bacterial cells can be classified by size, shape and cellular arrangement. In addition, bacteria can be further differentiated on the basis of their staining characteristics with Gram stain (Madigan, 200, Koneman et. al., 1997). Using this staining technique, most bacteria can be classified as gram-positive or gram-negative organisms. The Gram stain differentiates bacteria based on their cell wall structure.

The cell wall of gram-negative bacteria is a multilayered structure and quite complex, whereas the cell wall of gram-positive bacteria consists primarily of a single type of molecule and is often much thicker. The bacterial cell wall provides structural rigidity, confers shape to the cell, and forms a physical barrier against the outside environment. The rigid component of the cell wall of all bacteria is composed of peptidoglycan. The gram-positive bacteria cell wall is almost 80 nm thick and as much as 90% is composed mostly of peptidoglycan. Normally, peptidoglycan is ordered in several (up to about 25) layers (Figure 1).

In gram-negative bacteria, however, only about 10% of the wall is peptidoglycan. The majority of the wall consists of the outer membrane, which is made of lipopolysaccharide. This layer is effectively a second lipid bilayer, but it is not constructed solely of phospholipids, as is the cytoplasmic membrane ; instead, it also contains polysaccharide and protein. The lipid and

polysaccharide are linked in the outer layer to form specific lipopolysaccharide structures. Because of the presence of lipopolysaccharide, this outer layer is frequently called the lipopolysaccharide layer or LPS (Figure 2).

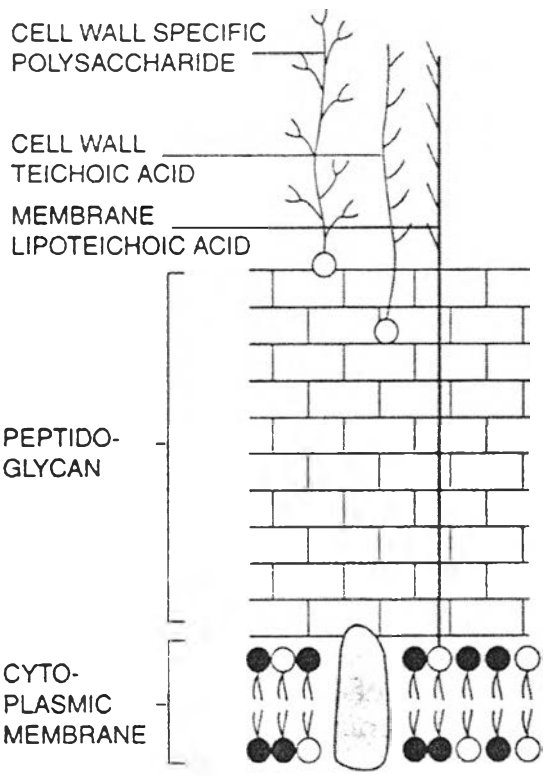

Figure 1 Structure of the cell wall of gram-positive bacteria (From Koneman et al., 1997).

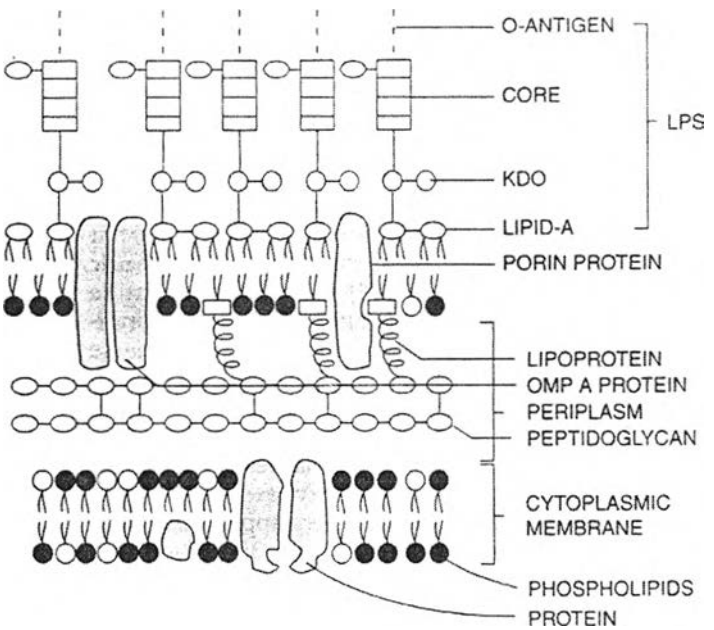

Figure 2 Structure of the cell wall of gram-negative bacteria. KDO, keto-deoxy-octulonate; LPS, lipopolysaccharide (From Koneman et al., 1997).

Endotoxin or lipopolysaccharide (LPS)

There are two types of bacterial toxins including exotoxin and endotoxin. Bacterial exotoxins are produced mostly by gram-positive bacteria. These proteins are released extracellularly as the organism grows but in some cases they are released by lysis of the bacterial cell.

Bacterial endotoxins, on the other hand, are cell-associated lipopolysaccharides (LPS), which are major components of the outer membrane of cell wall of gram-negative bacteria. They are the outermost structure encountering the surrounding environment. They are released from growing cells or from cells which are lysed as a result of effective host defense. The cell-associated LPS toxins are referred to as endotoxins. LPS molecules are complex glycolipids composed of a complex lipid portion called lipid A, a core polysaccharide region that is generally similar in structure within a given bacterial genus or species, and O-specific side chains, which are regions of variable biochemical structure.

The lipid A moiety of the LPS is embedded in the outer leaflet of the outer membrane, with the core polysaccharide and the O-specific side chains projecting from the outer membrane surface. Lipid A is composed of a glucosamine disaccharide. Attached to the lipid A portion of the LPS is the core polysaccharide. The core polysaccharide contains two unique carbohydrates : 2-keto-3-deoxyoctulonic acid (KDO), an 8-carbon sugar, and heptose, a 7-carbon sugar. The O-specific side chains are attached to the core polysaccharide and are responsible for the antigenic specificity of individual isolates. These side chains contain a variable number (up to about 40) of repeating oligosaccharide units composed of three to five monosaccharides each. The lipid A moiety appears to be the principal component responsible for the manifestations of endotoxic activity such as fever, hypotension and shock (Koneman et al., 1997, Todar, 2002) (Figure 3).

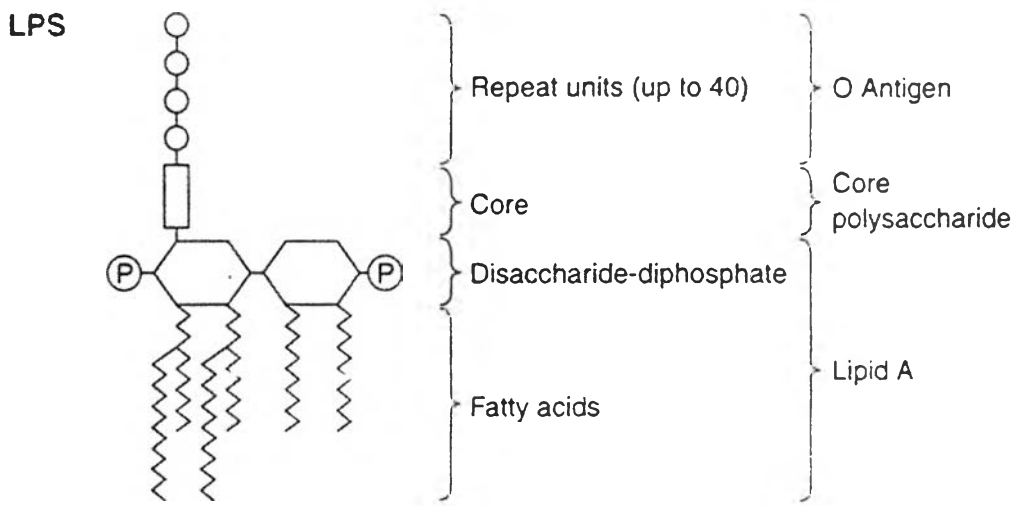

Figure 3 The lipopolysaccharide (LPS) of the gram-negative cell envelope: Segment of the polymer showing the arrangements of the major constituents (From Koneman et al., 1997).

LPS exerts its biological effects in an indirect manner. It stimulates host cells, mainly monocytes/macrophages, but also endothelial cells, smooth muscle cells, and neutrophils, to produce and release endogenous cytokines, such as interleukin-1 (IL-1), IL-6, and tumor necrosis factor-α (TNF-α). The presence of high amount of LPS leads to the large release of these mediators, resulting in the pathophysiological reactions (Schletter et al., 1995) (Figure 4). *In vitro*, LPS can stimulate adhesion molecule expression of the endothelial cells by stimulating cytokine and chemokine release from several cells types, including monocytes, macrophages, and endothelial cells (Mantovani et al., 1989, Pugin et al., 1993, Haziot et al., 1993, Schletter et al., 1995, Jiang et al., 2000, Chow et al., 1999, Pugin et al., 1993, Dentener et al., 1993). *In vivo*, LPS was able to stimulate endothelial adhesion molecule expression (Yipp et al., 2002) and leukocyte adhesion on endothelial cells (Woodman et al., 2000, Chakraphan et al., 2005).

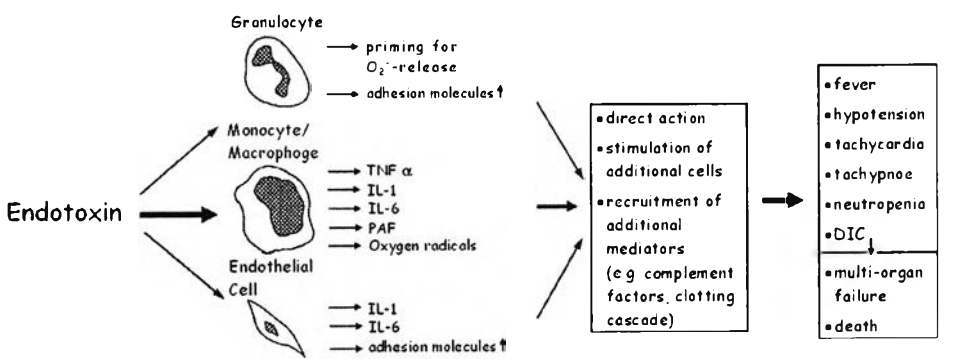

Figure 4 Scheme of the mode of action of LPS in the pathogenesis of septic shock. DIC disseminated intravascular coagulation (From Schletter et al.,1995).

Initial events in endothelial cell activation by LPS

LPS is a membrane lipid of Gram-negative bacteria that acts as a potent inflammatory stimulus in humans and other mammals. LPS stimulates cytokine and chemokine release from several cells types, including monocytes, macrophages, and endothelial cells. In the circulation, LPS is bound by lipopolysaccharide-binding protein (LBP) which is important in trafficking LPS. LBP facilitates the transfer of LPS to CD14, a glycoprotein found both as a soluble monomer in the blood (soluble CD14, sCD14) and as a glycosylphosphatidyl-inositol-linked membrane protein (mCD14) on monocytes and macrophages. CD14 acts as a carrier molecule, presenting LPS to a signaling receptor. Some evidence shows that members of the human Toll-like receptor (TLR) family are involved in CD14 dependent-LPS responses. Recent studies have identified TLR4 as a membrane cofactor in LPS-mediated transmembrane signaling in cytokine induction (Jiang et al., 2000, Chow et al., 1999, Yipp et al., 2002, Ishida et al., 2002). TLR4 is abundant on endothelial cell and is also expressed on monocytes and macrophage (Muzio et al., 2000). Normally, LPS induces endothelial cell responses by two different pathways, the direct pathway and indirect pathway (Mantovani et al., 1989, Pugin et al., 1993). The direct pathway requires the interaction of LPS with LBP and sCD14. After LPS is bound by LBP, LPS is transfered to sCD14. LPS-sCD14 complexes interact with TLR4 on the endothelial cell surface in order to trigger cytokine release which induce endothelial adhesion molecule expression and leukocyte adhesion (Haziot et al., 1993, Schletter et al., 1995). In the indirect pathway, LBP transfers LPS to mCD14 on macrophage or monocyte surface and LPS-mCD14 complexes interact with TLR4 to stimulate cytokine production and release, which in turn induce endothelial adhesion molecule expression and leukocyte adhesion (Chow et al., 1999, Jiang et al., 2000, Pugin et al., 1993, Dentener et al., 1993) (Figure 5).

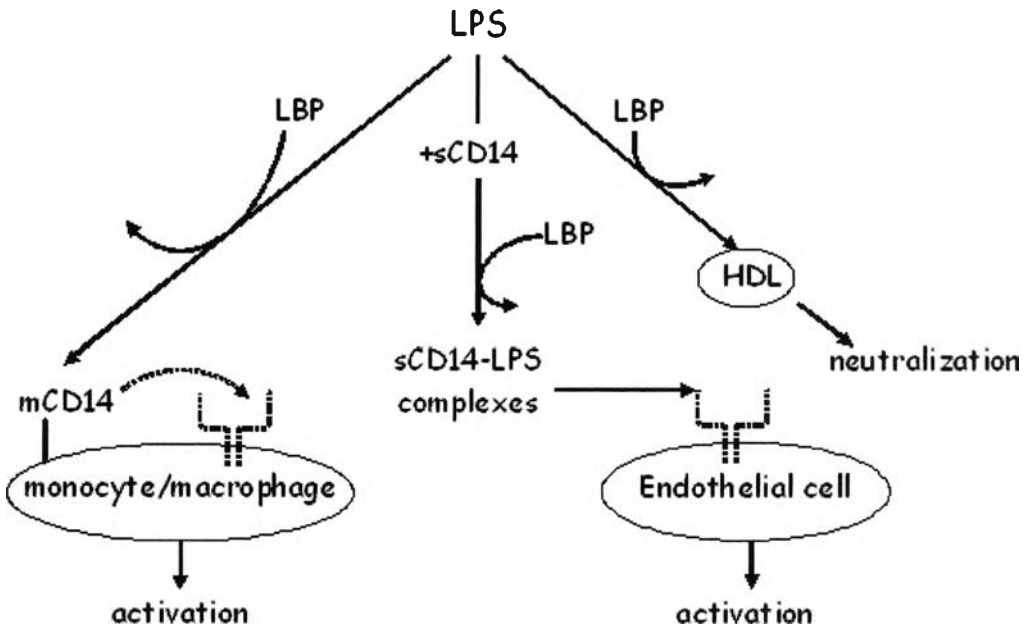

Figure 5 Initial events in cell activation by endotoxin. HDL high-density lipoprotein, LBP lipopolysaccharide-binding protein, mCD14 membrane bound CD14, sCD14 soluble CD14 (From Schletter et al.,1995).

Lipids and Lipoproteins (Mathews et al., 2000; Tuck, 2002)

The main lipids in human bodies are free cholesterol, cholesterol ester, triglyceride, and phospholipid. Triglyceride (TG) functions as the main form of long-term energy storage in the body. Cholesterol is an integral part of cell membranes, which is critical for membrane fluidity and function. Phospholipids are also important constituents of the cell membrane and play important roles in cell signaling. Both cholesterol ester and triglyceride are

nonpolar and immiscible in a water environment of plasma or serum. Therefore, cholesterol ester and triglyceride must be transported in the circulation by joining with proteins, called apolipoproteins, to form lipoproteins (Figure 6). Lipoproteins are spherical particles which contain a central core of nonpolar lipids (triglyceride and cholesterol esters), and a surface monolayer containing polar lipids (free cholesterol and phospholipids), and hydrophilic protein structures (Figure 7). Removal of lipids from lipoprotein by the process of delipidation results in a relatively pure mixture of proteins of HDL known as apoHDL.

Each class of lipoproteins (chylomicron, VLDL, IDL, LDL and HDL) is composed of different types of proteins and lipids, thereby responsible for different roles in lipid transport. Chylomicrons contain mostly TG and their main protein constituent is apolipoprotein B-48 (apo B-48). They carry dietary triglyceride to other tissues in the body and deliver nearly all the dietary cholesterol to the liver. VLDLs also contain mostly TG, some cholesterol/cholesterol ester and the main protein, apolipoprotein B-100 (apo B-100). They function to deliver TG and cholesterol made in the liver to peripheral tissues. IDLs are intermediates in the metabolic pathway between VLDL and LDL, whereas LDLs are the end products of a metabolic cascade. HDLs consist mainly of cholesterol/cholesterol ester, phospholipid and apolipoprotein A-I (apo A-I). HDL facilitates the movement of excess cholesterol derived from peripheral cells back to the liver for excretion (Table 1 & 2).

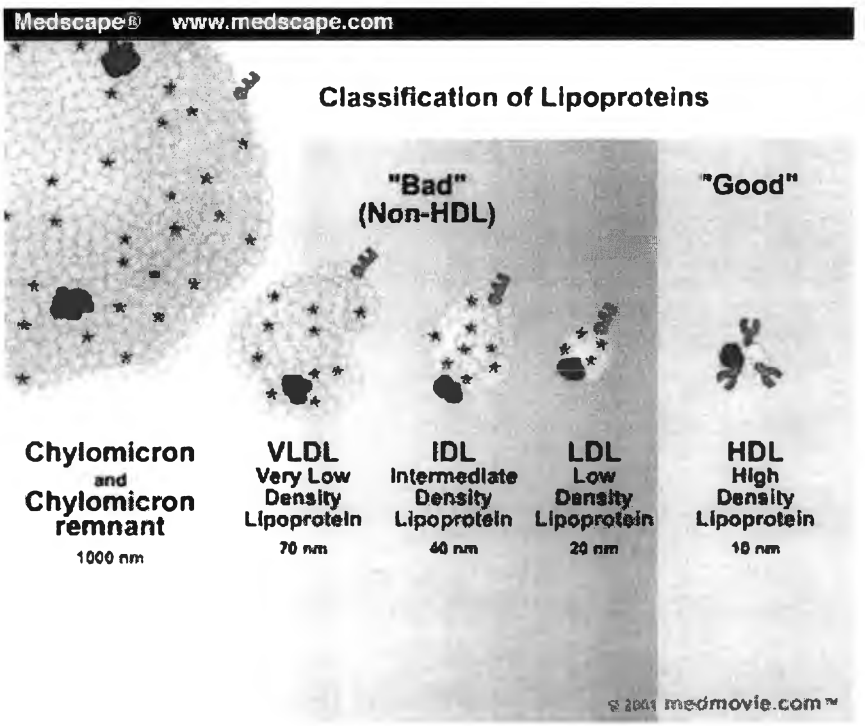

Figure 6 Classification of lipoproteins

(From http://www.medscape.com/viewprogram/608_index)

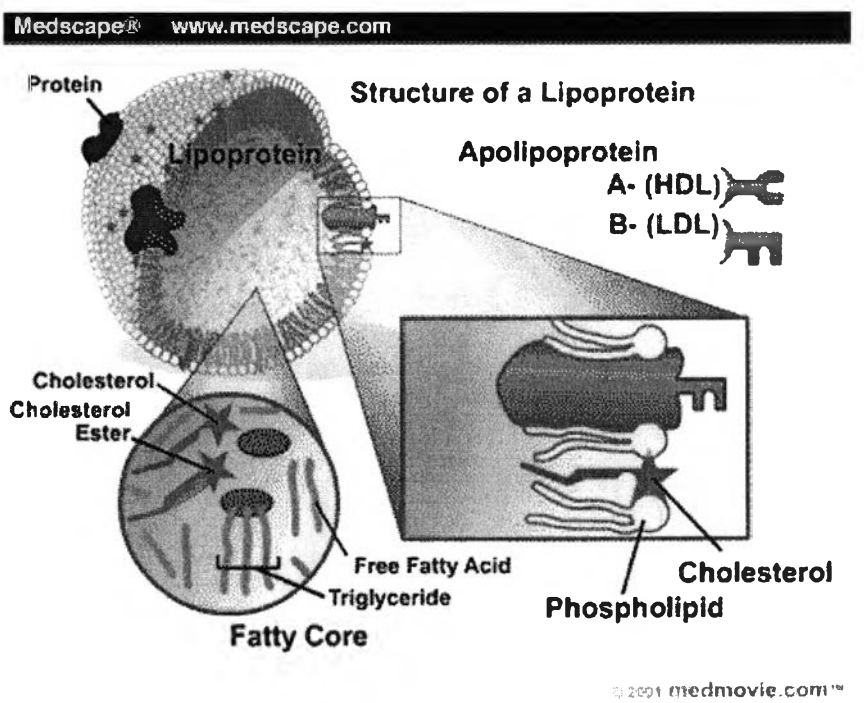

Figure 7 Structure of lipoprotein

(From http://www.medscape.com/viewprogram/608_index)

Table 1. Lipoprotein Classes

Lipoprotein class	Main lipid constituents	Main apolipoproteins	Hydrated density (g/mL)	Diameter (nm)
Chylomicrons	Triglyceride	Apo B-48	<0.95	100-1000
Chylomicron remnants	Triglyceride, cholesterol	Apo B-48, Apo E	<1.006	50-150
VLDL	Triglyceride	Apo B-100, Apo E	<1.006	35-50
IDL	Triglyceride, cholesterol	Apo B-100, Apo E	1.006-1.019	28-35
LDL	Cholesterol	Apo B-100, Apo E	1.019-1.063	20-26
HDL	Phospholipids, cholesterol	Apo A-I, Apo A-II	1.063-1.21	7-12
Lp(a)	Cholesterol	Apo B-100, Apo (a)	1.05-1.1	Variable

(From Tuck, 2002)

Table 2. Apolipoproteins

Apolipoprotein	Tissue of origin	Number of amino acids	lipoproteins	Plasma concentration (mg/L)
Apo A-I	Liver, intestine	243	HDL, chylomicrons	900-1600
Apo A-II	Liver	77	HDL, chylomicrons	250-450
Apo A-IV	Liver, intestine	376	HDL, VLDL, chylomicrons	100-200
Apo B-100	Liver	4,536	VLDL, LDL	500-1500
Apo B-48	Intestine	1,252	Chylomicrons	0-100
Apo C-I	Liver, intestine	57	Chylomicrons, VLDL, HDL	50-60
Apo C-II	Liver, intestine	79	Chylomicrons, VLDL, HDL	30-50
Apo C-III	Liver, intestine	79	Chylomicrons, VLDL, HDL	100-140
Apo D	Many tissues	169	HDL	40-70
Apo E	Most tissues	299	Chylomicrons, VLDL, HDL	20-80
Apo J	Most tissues	427	HDL	100
Apo (a)	Liver	variable	Lp(a)	Variable

(From Tuck, 2002)

Infection and the Acute-phase response (APR)

During infection and inflammation, there are a large number of changes and involving many organ systems. Accordingly, these systemic changes have been refered to as **the acute-phase response (APR),** although they accompany both acute and chronic inflammatory disorders. The acute-phase response, associated with infection, is part of host response which markedly affects the metabolism. The APR induces several behavioral and physiological responses such as fever, somnolence and anorexia (Table 3) (Gabay and Kushner, 1999). Biochemically, the APR is accompanied by changes in the concentration of specific plasma proteins, such as C-reactive protein, fibrinogen and albumin (Table 4). The increases in specific plasma proteins help protect the host from further injury and facilitate the repair process (Gabay and Kushner, 1999). Infection can also perturb lipoprotein metabolism and produce a wide variety of changes in the plasma concentrations of lipids and lipoproteins (Khovidhunkit et al., 2000).

Infection : Changes in Lipids/Lipoproteins : VLDL and LDL

Infection is associated with alterations in triglyceride and cholesterol metabolism. In addition to changes in circulating levels of lipids and lipoproteins, the composition of the lipoprotein particles is also altered. The most typical change in lipoprotein metabolism during infection is hypertriglyceridemia (Cabana et al., 1989; Grunfeld et al., 1992; Khovidhunkit et al., 2000). An increase in plasma triglyceride levels is due to an increase in VLDL, which can be the result of either increased VLDL production or decreased VLDL clearance (Table 5).

In contrast, LDL and Apo B-100 levels are decreased in patients with infection (Grunfeld et al., 1992). LDL isolated from animals treated with LPS is enriched in certain sphingolipids such as ceramides and sphingomyelin (Memon et al., 2000).

Infection : Changes in HDL levels, composition and functions

Infection is associated with a decrease in HDL cholesterol levels (Cabana et al., 1989; Grunfeld et al., 1992; Feingold et al., 1993; Khovidhunkit et al., 2000). Lipid and protein composition of HDL is markedly affected which results in alterations in various functions of HDL. Circulating HDL that occurs during infection is called **Acute-phase HDL (AP-HDL)**. AP-HDL contains more free fatty acids and free cholesterol. In addition, the protein component of AP-HDL is also altered. AP-HDL is enriched in serum amyloid A (SAA), the major acute-phase protein (Hoffman and Benditt, 1982; Cabana et al., 1989; Gabay and Kushner, 1999). SAA has been reported to cause adhesion and chemotaxis of phagocytic cells and lymphocytes and may contribute to inflammation in atherosclerotic coronary arteries by increasing the oxidation of LDL (Gabay and Kushner, 1999). Several HDL-associated proteins have the antioxidant effects. During infection, these HDL-associated proteins are decreased which cause the loss of antioxidant effects of AP-HDL. Therefore, AP-HDL is less effective in preventing LDL oxidation (Ehrenwald et al., 1994, Van Lenten et al., 1995) (Table 5).

Not only alteration in the antioxidative effects of AP-HDL occurs but the role of AP-HDL in reverse cholesterol transport is also disturbed. Apo A-I, the major protein of HDL, is decreased during infection. Therefore, apolipoprotein-mediated cholesterol removal from cells is impaired. Furthermore, other protein compositions of AP-HDL are also changed which affects the cholesterol movement out of the cells. This effect could also be potentially atherogenic (Tall, 1986; Cabana et al., 1989; Grunfeld et al., 1992; Khovidhunkit et al., 2000; Khovidhunkit et al., 2001; Barter, 2002).

Table 3. Other Acute-Phase Phenomena.

Neuroendocrine changes
Fever, somnolence, and anorexia
Increased secretion of corticotropin-releasing hormone, corticotropin, and cortisol
Increase secretion of arginine vasopressin
Decreased production of insulin-like growth factor I
Increased adrenal secretion of catecholamines
Hematopoietic changes
Anemia of chronic disease
Leukocytosis
Thrombocytosis
Metabolic changes
Loss of muscle and negative nitrogen balance
Decrease gluconeogenesis
Osteoporosis
Increased hepatic lipogenesis
Increased lipolysis in adipose tissue
Decrease lipoprotein lipase activity in muscle and adipose tissue
Cachexia
Hepatic changes
Increased metallothionein, inducible nitric oxide synthase, heme oxygenase, manganese superoxide dismutase, and tissue inhibitor of metalloproteinase-I
Decreased phosphoenolpyruvate carboxykinase activity
Changers in nonprotein plasma constituents
Hypozincemia, hypoferremia, and hypercupremia
Increased plasma retinol and glutathione concentrations

(From Epstein, 1999)

Table 4. Human Acute-Phase Proteins.

Proteins whose plasma concentrations increase
Complement system
C3
C4
C9
Factor B
C1 inhibitor
C4b-binding protein
Mannose-binding lectin
Coagulation and fibrinolytic system
Fibrinogen
Plasminogen
Tissue plasminogen activator
Urokinase
Protein S
Vitronectin
Plasminogen-activator inhibitor 1
Antiproteases
α_1-Protease inhibitor
α_1-Antichymotrypsin
Pancreatic secretory trypsin inhibitor
Inter—trypsin inhibitors
Transport proteins
Ceruloplasmin
Haptoglobin
Hemopexin
Participants in inflammatory response
Secreted phospholipase A_2
Lipopolysaccharide-binding protein (LBP)
Interleukin-1-receptor antagonist
Granulocyte colony-stimulating factor
Others
C-reactive protein
Serum amyloid A
α_1-Acid glycoprotein
Fibronectin
Ferritin
Angiotensinogen
Proteins whose plasma concentrations decrease
Albumin
Transferrin
Transthyretin
A_2-HS glycoprotein
Alpha-fetoprotein
Thyroxine-binding globulin
Insulin-like growth factor I
Factor XII

(From Epstein, 1999)

Table 5. Potential proatherogenic changes and effects of lipoproteins during infection and inflammation.

Changes	Effects
VLDL	
Increased VLDL levels	Provides lipid substrates for macrophage uptake
Decreased LPL and HL	Decreases clearance of triglyceride-rich lipoproteins
Increased sphingolipid content	Decreases clearance of triglyceride-rich lipoproteins
Decreased tissue apo E expression	Decreases lipoprotein clearance
LDL	
Increased small dense LDL	Increases susceptibility to oxidation; increase LDL penetration through endothelium; increase interaction with arterial wall proteoglycans and LDL retention in arterial wall
Increased PAF-AH activity	Increases LPC production
Increased $sPLA_2$	Releases polyunsaturated fatty acids from phospholipids that can become oxidized fatty acids
Increased sphingolipid content	Facilitates LDL aggregation and uptake into macrophages
Increased ceruloplasmin	Increases LDL oxidation
HDL	
Decreased HDL and apo A-I	Impairs apolipoprotein-mediated cholesterol removal from cells
Decrease LCAT	Impairs cholesterol removal from cells by diffusion mechanism
Decreased CETP	Impairs cholesterol transfer to triglyceride-rich lipoproteins
Decreased HL	Reduces pre-β HDL generation
Decreased PLTP	Reduces pre-β HDL generation; decrease HDL phospholipid content and impairs cholesterol removal by increasing cholesterol flux from HDL into cells
Increased SAA	Decreases availability of cholesterol in HDL to be metabolized by hepatocytes; increases cholesterol uptake into macrophages
Increased sPLA2	Decreases HDL phospholipid content and impairs cholesterol removal by increasing cholesterol flux from HDL into cells
Increased PAF-AH activity	Increases LPC production
Decreased PON	Decreases ability of HDL to protect against LDL oxidation
Decreased transferrin	Impairs the ability of HDL to prevent LDL oxidation
Increased apo J	Induces smooth muscle cell differentiation in arterial wall

Note. Apo, apolipoprotein; CETP, cholesterol ester transfer protein; HDL, high-density lipoprotein; HL, hepatic lipase; LCAT, lecithin:cholesterol acyltransferase; LDL, low-density lipoprotein; LPC, lysophosphatidylcholine; LPL, lysophosphantidylcholine; LPL, lipoprotein lipase; PAF-AH, platelet-activating factor acetylhydrolase; $sPLA_2$, secretory phospholipase A_2; PLTP, phospholipid transfer protein; PON, paraoxonase; VLDL, very low-density lipoprotein; SAA, serum amyloid A.

(From Khovidhunkit et al., 2000)

HDL and antibacterial activity

Although HDL is well known in its role in protecting against atherosclerosis, the role of HDL in innate immunity is lesser known.

HDL can bind LPS which converts LPS to a form with a buoyant density of less than 1.2 g/cm^3. The reduction in its buoyant density is accompanied by a loss of toxic effects of LPS (Ulevitch and Johnston, 1978; Weinstein and Ulevitch, 1979; Ulevitch et al., 1979; Ulevitch et al., 1981). It has been proposed that the lipid-A domain of LPS may be masked by insertion into the phospholipid bilayer of HDL, resulting in the detoxification of LPS (Levine et al., 1993). In addition, failure of LPS to induce fever can be demonstrated using pre-incubation of LPS with HDL or apo A-I (Emancipator et al., 1992). Moreover, reconstituted HDL was a potent inhibitor of LPS-induced tumor necrosis factor-α production in human whole blood (Parker et al., 1995). In addition, LPS-induced interleukin-1β and tumor necrosis factor-α release from peripheral blood mononuclear cells decreased after LPS was preincubated with lipoproteins (Sprong et al., 2004). HDL also has anti-inflammatory properties *in vivo*. Raising the plasma HDL concentration protected mice against endotoxin *in vivo*. Transgenic mice with 2-fold elevated plasma HDL levels had more endotoxin bound to HDL, lower plasma cytokine levels, and improved survival rates compared with control mice. Intravenous infusion of reconstituted HDL or HDL apoprotein also protected mice from endotoxin (Levine et al., 1993). In hypolipidemic models, LPS produced a greater increase in serum tumor necrosis factor levels than it did in controls, and administration of exogenous total lipoproteins prevented this increase that tumor necrosis factor was responsible for most of the toxic effects of LPS (Feingold et al., 1995). It has been postulated that lipid A component, the moiety causing cytokine activation and the most hydrophobic part of LPS, could be bound and sheltered by the hydrophobic structures of apo A-I (Flegel et al., 1993). HDL can also bind and neutralize the toxic effect of lipoteichoic

acid (LTA), a cell wall component of gram-positive bacteria (Grunfeld et al., 1999). Moreover, reconstituted HDL could diminish cytokine production upon stimulation with LPS and clinical symptoms in humans (Pajkrt et al., 1996).

HDL also has effects on adhesive proteins and endothelial cells. Infusion of reconstituted HDL and apo A-I inhibited the infiltration of neutrophils, and the expression of adhesion molecules on endothelial cells induced by a periarterial collar in normocholesterolemic rabbits (Nicholls et al., 2005). The inhibitory effects of HDL on cytokine-induced endothelial cell adhesion molecule expression were found in several *in vitro* studies (Ashby et al., 1998, Ashby et al., 2001, Cockerill et al., 1995, Cockerill et al., 2001, Baker et al., 1999). *In vivo*, elevating HDL in the circulation led to inhibition of cytokine-induced E-selectin expression in porcine microvascular endothelial cells (Cockerill et al., 2001).

Not only is HDL able to inactivate the toxic effects of LPS and LTA, but it can also affect the living bacterial cells. Human HDL inhibits the growth of *S.epidermidis in vitro* (Tada et al., 1993). It is possible that the insertion of lipids or proteins of HDL into the bacterial cell membrane can inhibit the growth of living bacteria and results in the cell lysis (Tada et al., 1993). In fact, HDL has been shown to inhibit the growth of *Trypanosome protozoa* by inducing cell lysis (Rifkin, 1991). However, mechanism of HDL-induced cell lysis did not result from insertion of proteins of HDL into cell membrane but was due to forming anion channels in the lysosomes membrane resulting in osmotic swelling and subsequent cell lysis (Perez-Morga et al., 2005).

In vivo studies of effects of HDL on LPS-induced leukocyte adhesion on endothelial cells and mesenteric intravital microscopy

LPS acts as the inflammatory stimulus. The inflammatory response is characterized by an infiltration of leukocytes, the major cellular defense mechanism. The multistep inflammatory process involves leukocyte rolling,

adhesion, and emigration on the post-capillary venule endothelial cell, because the post-capillary venules appear to have the highest density of receptors for inflammatory mediators (Gavins et al., 2004) prior to the migration into the site of tissue injury (Figure 8). The first three steps of these processes can be studied by intravital microscopy (Gavins et al., 2004). Intravital microscopy is an useful qualitative and quantitative technique for study leukocyte-endothelium interaction *in vivo*. Since its first use, intravital microscopy has been used in a variety of microcirculatory preparations including the bat wing, hamster cheek pouch, rabbit ear, rodent mesentery, and cremaster (Gavins et al., 2004). The mesentery is a translucent tissue. This tissue can be easily inflamed by the injection of various stimuli, such as LPS, into the peritoneal cavity (Brito et al., 1998).

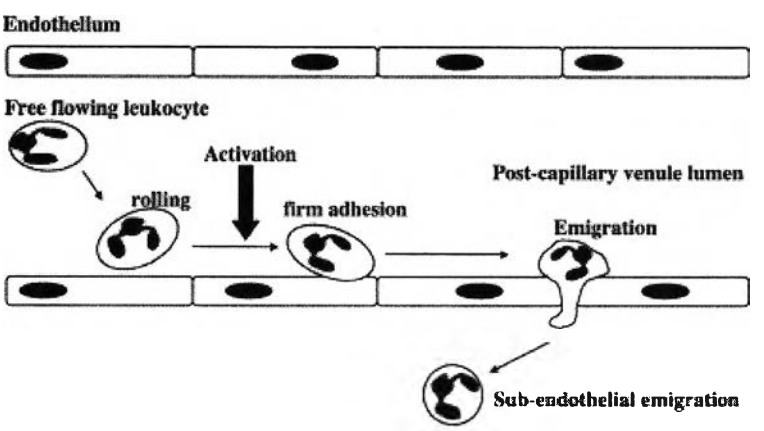

Figure 8 Schematic diagram of the multistep process of leukocyte-endothelial cell interactions. Diagram showing the main processes of rolling, firm adhesion, and emigration in a post-capillary venule (From Gavins et al., 2004).

From the above evidence, it was hypothesized that normal HDL and AP-HDL could inhibit the growth of both gram-negative and gram-positive bacteria. Beside the inhibitory effects on the living bacteria, HDL might also affect the toxic effects of LPS. Therefore, this present study examined whether

normal HDL and AP-HDL could inhibit the growth of gram-negative and gram-positive bacteria *in vitro*. In addition, we examined whether normal HDL and AP-HDL could inhibit LPS-induced leukocyte adhesion on the endothelium *in vivo* using intravital microscopy.