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Review Article

Bacterial endotoxin-lipopolysaccharide; structure, function and its role in immunity in vertebrates and invertebrates

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ABSTRACT

Biotic and abiotic factors shape investment in costly defenses. The immune systems of vertebrates and invertebrates differ in their fitness cost. However, the hygienic behavior of both can result in increased survival rate and fitness cost. The immune response of vertebrates has developed more sophisticated and complicated mechanisms including an immunological memory with the generation of large antigen-recognition receptors and innate immune systems. The invertebrate immune system must rely on non-self-recognition molecules to ensure efficient defense responses against infectious pathogens that continuously threaten their survival. Lipopolysaccharide (LPS) from bacterial endotoxin, has been regarded as having potential molecules involved in immune recognition and immune defense. This review focused on an overview of bacterial endotoxin, LPS, and their structure, function, and elucidation of immune responses in both vertebrates and invertebrates are discussed. In addition, invertebrate defense against LPS is reviewed in detail. The precise mechanisms underlying self and non-self-recognition represent the basis to prevent and control infections from endotoxins as well as to stimulate animal resistance. This is particularly relevant for immune defense against LPS in invertebrates and vertebrates which are frequently constrained by recurrent diseases.

Introduction

Lipopolysaccharide (LPS) is the major constituent of the outer membrane of all Gram-negative bacteria and this is the bacterial-associated substance called endotoxin that elicits septic shock in animals (Beutler and Rietschel, 2003). Immune systems have developed to protect multicellular organisms from self or foreign "nonself" substances. During evolution, two general immune systems have developed to detect foreign substances namely innate (natural) immunity and adaptive (acquired) immunity. The innate immune system is phylogenetically a more ancient defense mechanism and can be found in all multicellular organisms. This system is the first-line of host defense that helps to limit infection in the early stage of infection and relies on germ line-encoded receptors that recognize conserved molecular patterns found in microorganisms (Fearon and Locksley, 1996; Fearon, 1997; Medzhitov and Janeway, 1997; Vishnu Priya, 2015). It is now clear that the innate immune system is very important for self or non-self recognition in vertebrates and plays an important role in adaptive immune systems (Medzhitov and Janeway, 1998a; Vishnu Priya, 2015). Various cells in invertebrates respond to microorganisms by enclosing these infectious agents within aggregates and then destroying them. The innate immune system of invertebrates can respond to the presence of pathogens with cellular and humoral responses (Vishnu Priya, 2015). One of the most abundant sources of LPS encountered by vertebrates is their resident gut microbiota and intestinal alkaline phosphatase detoxify the LPS and prevent intestinal inflammation in response to the resident microbiota (Bates et al., 2007).

Structure of cell wall of Gram-negative bacteria

In Gram-negative bacteria, one of the major important components is endotoxin, which is present in the outer membrane of the cell wall. The cell envelope of Gram-negative bacteria (Fig. 1) consists of the inner membrane (IM), the peptidoglycan (murein) and the outer membrane (OM) (Raetz and Whitfield, 2002). The IM is a phospholipids bilayer, which is similar to the plasma membrane of eukaryotic cells, and is permeable to lipophilic compounds. In 1892, Richard Pfeiffer first defined endotoxin as a heat-stable, toxic substance that was released upon disruption of microbial envelopes (Beutler and Rietschel, 2003). Numerous integral

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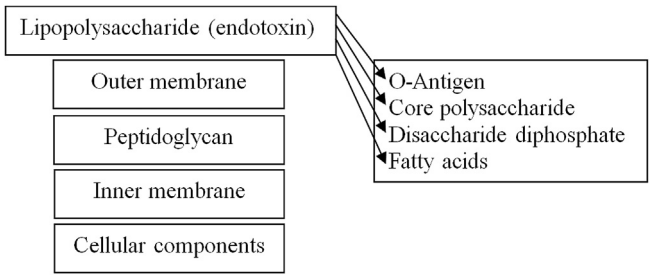


Fig. 1. Basic components of bacterial endotoxin.

transmembrane, helical proteins and peripheral membrane proteins are primarily responsible for transport, cell signaling and metabolic functions (Harald, 2001). The periplasm is the gelatinous material between the outer membrane and the IM. It contains enzymes for nutrient breakdown as well as binding proteins to facilitate the transfer of nutrients across the IM. Peptidoglycan in the periplasmic space is composed of alternating N-acetyl glucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc) sugars that are cross-linked by short peptide bridges and maintains the osmotic pressure and cell structure (Holtje, 1998). The outer membrane is unique to Gram-negative bacteria, and its role is to serve as a protective structure. The lipid structures are highly asymmetric. LPSs are arranged in a tightly packed structure in the outer membrane (Kamio and Nikaido, 1976; Vishnu Priya, 2015).

Structure of LPS

The term endotoxin refers to cell-associated toxin and based on their internal compounds it generally refers to lipopolysaccharide (LPS). This LPS is composed of two major important components, hydrophilic lipid A and hydrophilic polysaccharide (O-region). Both are important for endotoxin biological activity. Toxicity is associated with the lipid component (Lipid A) and immunogenicity is associated with the polysaccharide components. The cell wall antigens (O antigens) of Gram-negative bacteria are components of LPS. LPS elicits a variety of inflammatory responses in an animal and it activation is complemented by the alternative (properdin) pathway; hence, it may be a part of the pathology of Gram-negative bacterial infections (Gutsmann et al., 2005; Vishnu Priya, 2015).

LPS has a molecular weight >100,000 D. The lipid A portion of the molecule has been shown to be responsible for numerous *in vivo* and *in vitro* effects of endotoxin. LPS stimulates the immune responses (Yu and Kanost, 2004) and enhances cellular immune reactions (Foukas et al., 1998; Soldatos et al., 2003). However, it has been reported that commercial bacterial endotoxin LPS contains enough peptidoglycan (PGN) to activate antimicrobial peptide (Dziarski and Gupta, 2006). Low activities of endotoxin stimulate the immune response and higher activities can lead to septic shock. *In vivo*, Gram-negative bacteria probably release minute amounts of endotoxin while growing. This may be important in the stimulation of natural immunity. Endotoxins are heat stable (boiling for 30 min does not destabilize endotoxin), but not to certain powerful oxidizing agents such as superoxide, peroxide and hypochlorite, which are used to remove the bacterial endotoxin (Vishnu Priya, 2015).

Detailed view of lipopolysaccharide components

Lipid A is the lipid component of LPS. It contains the hydrophobic, membrane-anchoring region of LPS. Lipid A consists of a phosphorylated N-acetyl glucosamine dimer with 6 or 7 fatty acids attached, usually six fatty acids are found. All fatty acids in Lipid A are saturated. Some fatty acids are attached directly to the N-acetyl glucosamine dimer and others are esterified to the 3-hydroxy fatty acids that are characteristically present. The structure of Lipid A is highly conserved among Gram-negative bacteria (Vishnu Priya, 2015).

Core (R) antigen or R polysaccharide is attached to the six position of one NAG. The R antigen consists of a short chain of sugars. For example: KDO – Hep – Hep – Glu – Gal – Glu – GluNAc. Two unusual sugars, heptose and 2-keto-3-deoxyoctonoic acid (KDO), are usually present in the core polysaccharide. KDO is unique and invariably present in LPS and so it has been used as an indicator in assays for LPS (endotoxin) (Vishnu Priya, 2015).

Vishnu Priya (2015) reported that somatic (O) antigen or O polysaccharide is attached to the core polysaccharide. It consists of repeating oligosaccharide subunits made up of 3–5 sugars. The individual chains vary in length ranging up to 40 repeat units. The O polysaccharide is much longer than the core polysaccharide, and it maintains the hydrophilic domain of the LPS molecule. A major antigenic determinant (antibody-combining site) of the Gram-negative cell wall resides in the O polysaccharide. The cecropins are antimicrobial peptides which have a broad spectrum of activity against Gram-positive and Gram-negative bacteria (Suparna et al., 2011).

Major variation occurs in the composition of the sugars in the O side chain between species and even strains of Gram-negative bacteria. At least 20 different sugars are known to occur and many of these sugars are characteristically unique dideoxyhexoses, which occur in nature only in Gram-negative cell walls. Variations in sugar content of the O polysaccharide contribute to the wide variety of antigenic types of *Salmonella* and *E. coli* and mostly other strains of Gram-negative species. Defined sugars in the structure, especially the terminal ones, elicit immunological specificity of the O antigen. Smooth strains (S-Strain) are produced by the presence of O part whereas the rough strains (R-Strain) are produced by the absence of the O region (Vishnu Priya, 2015).

Virulence/Toxicity

Both Lipid A (the toxic component of LPS) and the polysaccharide side chains (the nontoxic but immunogenic portion of LPS) act as determinants of virulence in Gram-negative bacteria. O-antigens have adhered properties and these are resistance to phagocytes, protection toward to antigens and antigenic variation property. Lipid A act as an immune stimulator, which induces the biological responses of a specific organism (Hancock and Diamond, 2000; Papo and Shai, 2005; Vishnu Priya, 2015).

Biological activity of lipopolysaccharide

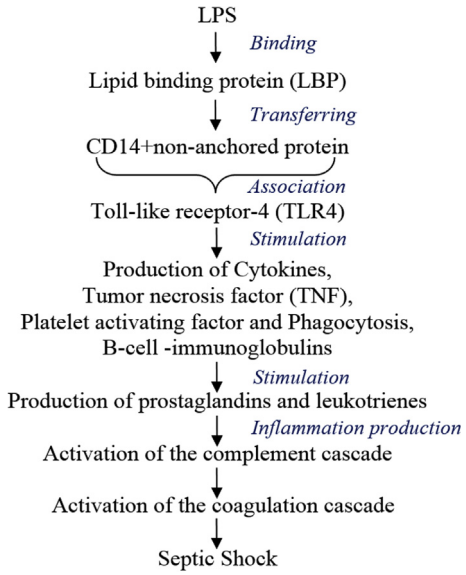
An experimental animal's biological immune responses may be analyzed using several parameters, like injection of living or killed Gram-negative cells or purified LPS into experimental animals causes a wide spectrum of nonspecific pathophysiological reactions, such as fever, changes in white blood cell counts, disseminated intravascular coagulation, hypotension, shock and death. The injection of minimum doses of endotoxin results in death in most mammals. The sequence of events follows a regular pattern: (1) latent period; (2) physiological distress (diarrhea, prostration, shock); and (3) death. How soon death occurs varies on the dose of the endotoxin, the route of administration, and the species of animal. Animals vary in their susceptibility to endotoxin. The recent development of transgenic technologies and silkworm genome

information are expected to accelerate silkworm immunity studies (Gillespie et al., 1997; Tanaka and Yamakawa, 2011).

Dosage-dependent lipopolysaccharide activity

Ratcliffe and Gagen (1976) analyzed pathogen dosage-dependent activity. They studied the *in vivo* cellular activity between bacteria and *Galleria mellonella* and also in *Pieris brassicae* larvae (1976). Similar dosage-dependent activity was reported in *Galleria mellonella* (DeVemo et al., 1983; Schwalbe and Boush, 1971) and also in *Spodoptera eridiana* larvae (Anderson, and Cook, 1979). In addition, dosage dependency with a pattern recognition process in response to LPS was reported in *Estigmene acraea* by Wittwer et al. (1997). Similarly, dosage-dependent activity under LPS in silkworm had been reported by Vishnu Priya (2015).

Lipopolysaccharide signaling and immune activation mechanism in higher organism



Lipopolysaccharide detoxification mechanism in higher animals

In vertebrates, defense against infection is mediated by two immune systems known as innate and adaptive. The adaptive immune system has the ability to recognize a wide spectrum of antigens through a diverse array of somatically rearranged receptors (T-cell and B-cell receptors) Royet (2004). The eradication of LPS biological activity is called detoxification (Ulevitch and Johnston, 1978); the direct interactions of bacterial LPS with humoral as well as cellular elements may lead to the production of injurious mediators responsible for the initiation of LPS-induced hemodynamic and coagulative changes (Ulevitch and Johnston, 1978) and followed by pathophysiological changes in the host, so, therefore, the host will interact with LPS and the specificity binding progress process occurs. The production of humoral and cellular component stimulation may lead to a loss of biological activity of LPS (Ulevitch and Johnston, 1978). The mechanism of LPS detoxification occurs in two ways, either by enzymatic degradation or by complement-mediated detoxification subject to disaggregation of LPS. It has been proven that the purified and native forms of LPS differ in their specific activity.

Host-microbe interactions (lipopolysaccharide activity) in invertebrates-insects

An insect's innate immune system plays an important role in the development of immunity (Hoffmann, 2003). In recent years, arthropods and insects have therefore been very useful models to dissect the molecular regulation of the innate immune response (Royet, 2004). Insects possess highly effective defense mechanisms against invading microorganisms involving Gram-negative, Gram-positive, LPS and peptidoglycan among others (Hultmark, 1993; Hoffmann et al., 1996; Hoffmann and Reichhart, 1997).

These defense mechanisms involve both cellular and humoral responses. The cellular response includes phagocytosis and/or nodule formation of bacteria and encapsulation of larger parasites by blood cells (hemocytes) (Lackie, 1988). On the other hand, the humoral response utilizes various antimicrobial peptides synthesized in the fat body and some hemocytes upon induction by septic injury and which are then secreted into the hemolymph (Cociancich et al., 1994; Meister et al., 1997; Koizumi et al., 1999). Insect defense system against LPS pathogens comprises phagocytosis and encapsulation of invaders by the hemocytes and the subsequent production of antimicrobial proteins (mainly in the insect fat body), which results in a temporary increase of antimicrobial activity in the cell-free hemolymph (Wittwer et al., 1997). Strong immune activity was found in the interaction between *Galleria mellonella* and LPS. LPS acts as an immune stimulator for subsequent administration of Gram-negative bacteria. The high LPS-resistance may be explained by a very efficient detoxification mechanism which was reported to involve the binding of LPS by hemolymph lipophorins (Kato et al., 1994). This proved that LPS has pre immune activation ability.

In general insects hemocytes plays a main role to induce immunity against a pathogenic compound. Activation of a prophenol oxidase cascade induced by a pathogenic compound (β 1,3 glucans/ LPS) binds with cray fish and the immune reaction in the form of degranulation of crayfish hemocytes (Soderhall et al., 1996). In most of the insects, immuno proteins (or) AMP are involved in LPS and β1,3-glucan-dependent activation of the protease cascades leading to prophenoloxidase activation (Royet, 2004; Sugumaran and Kanost, 1993). In most cases, *GNBP* genes are expressed in immune tissues such as the fat body and hemocytes and their transcription is unregulated when challenged (Kim et al., 2002; Ma et al., 2007).

Activation of the proteolytic cascade and clotting cascade with the help of LPS triggers the Limulus hemocyte in the action of a signaling mechanism (Kawabata et al., 1996). In addition, similar work was reported in *Bombyx mori* by Ochiai and Ashida (1988 & 2000). Furthermore, the hemocyte membrane receptor for LPS that may transduce the activation signal for the synthesis of the antibacterial peptide cecropin B has been isolated from the insect *Bombyx mori* (Xu et al., 1995). Additional important functions of hemocytes lie in their ability to release immune-stimulating factors during cellular defense reactions (Wiesner et al., 1996). Similarly, the synthesis of the antimicrobial protein cecropin was reported in Drosophila (Hultmark, 1994); Kim et al., 2002). LPS induces the loss of function in drosophila phenotypes and AMP regulation in RNA has been observed (Leulier et al., 2003; Werner et al., 2000, 2003; Gottar et al., 2002; Choe et al., 2002; Takehana et al., 2002).

In addition, phagocytosis and lysosome production under LPS in *Estigmene acraea* was reported by Wittwer et al. (1997). Additionally, LPS-dependent induction of antimicrobial activity and the influence on development and mortality was investigated in the greater wax moth *Galleria mellonella*. LPS-induced immune responses were reported by Royet (2004). The c-terminal part of *Bombyx mori* has a strong specificity and higher affinity for LPS. However, LPS is unable to activate the immune response in flies (Leulier et al., 2003).

LPS-mediated cellular aggregation reaction was observed in *Plodia interpunctella* (Fabrick et al., 2003). Similarly, it was reported with prophenol oxidase activation in *Manduca sexta* (Yu and Kanost, 2000). Molecular cloning and characterization of phenoloxidase under pathogens was reported in *Penaeus monodon* (Sritunyalucksana et al., 1999). The American cockroach *Periplaneta americana* has two LPS recognition proteins: a lipopolysaccharide-binding protein (LBP) (Jomori et al, 1990, Jomori and Natori, 1991, 1992) and *Periplaneta* lectin (Kubo and Natori, 1987; Kawasaki et al., 1993). Hemolin, a member of the immunoglobulin super family, has been identified in the giant silk moth *Hyalophora cecropia* and the tobacco hornworm *Manduca sexta* (Sun et al., 1990; Ladendorff and Kanost, 1991). The lipid A portion of LPS stimulates phagocytosis (Daffre and Faye, 1997; Lanz-Mendoza et al., 1996). LPS-binding protein (BmLBP) was found in silkworm for clearance of Gram-negative bacteria strains from the hemolymph (Koizumi et al., 1997, 1999).

Expression of genes and signaling action induced by lipopolysaccharide in vertebrates and invertebrates

It is a general phenomenon that antibacterial gene expression reaches its maximum level a few hours after bacterial infection and gradually declines with time. This decrease in antibacterial protein gene expression was shown to be related with the LPS clearance mechanism. The Toll-like receptors (TLRs), a class of PRRs found in vertebrates, play a key role not only in the initiation of innate immunity but also in the activation of acquired immunity (Takeda and Akira, 2005). Peptidoglycan recognition proteins (a novel family of innate immunity pattern recognition molecules) was observed and identified in humans (Liu et al., 2001). A peptidoglycan recognition protein in innate immunity was conserved from insects to humans (Kang et al., 1998).

LPS non-specific recognition is well documented and reported in insects (Fabrick et al., 2003; Ma and Kanost, 2000, 2001; Zhang et al., 2003). The signaling and triggering mechanism was identified in *Bombyx mori* (Ochiai and Ashida, 1988; Yoshida et al., 1986). In Drosophila, signal peptide expression is via secreted proteins (Kim et al., 2000). The structural and functional similarities between the Toll and the TLR-dependent activation of NF−kB has been interpreted as evidence for the existence of a common ancestor and shared mechanisms between the vertebrate and invertebrate innate immune systems (Royet, 2004). LPS-increased levels of mRNAs were observed in *A. gambiae* of a *Plasmodium berguei*-infected blood-meal (Dimopoulos et al., 1997, 1998). LPS and β-1,3-glucan binding protein (LGBP) were isolated from *Litopenaeus vannamei* in which mRNA expression was induced by the challenge of bacteria *Vibrio alginolyticus* (Yeh et al., 2009). Similarly, phenoloxidase activity was analyzed and characterized in *Armigeres subalbatus* (Cho et al., 1998), *Drosophila melanogaster* (Werner et al., 2000), *Macrobrachium rosenbergii* (Liu et al., 2006a), *Charybdis japonica* (Liu et al., 2006b), *Pacifastacus leniusculus* (Liu et al., 2007a), *Fenneropenaeus chinensis* (Liu et al., 2007b), *Tenebrio molito* (Liu et al., 2007c) and diamondback moths (Liu et al., 2009).

Temperature-dependent phenol oxidase activity was analyzed with LPS in *Litopenaeus vannamei* (Pan et al., 2008). Melanization reactions were linked with bacterial infections in *Anopheles gambiae* (Schnitger et al., 2007). In addition, BmLBP was expressed in multiple different tissues with distinct expression patterns (Hinako Takase et al., 2009). In supporting the view of above, analysis of dot and Northern blot hybridization revealed that the hemocytin gene is transcribed in hemocytes of the silkworm *Bombyx mori* at the larval-pupal metamorphosis and/or after injection of *Escherichia coli* and LPS.

This review has highlighted the main features of the bacterial endotoxin structure, function and their role in the development of immunity in both vertebrates and invertebrates. In the detailed review above, vertebrates were shown to have acquired immunity with 'immunological memory', whereas invertebrates lack this immune system. Instead, they possess innate immunity, which is characterized by non-specific immune reactions against foreign materials. Invertebrates possess highly effective defense mechanisms of both cellular and humoral reaction against bacterial infections (Hultmark, 1993; Hoffmann and Reichhart, 1997; Hoffmann et al., 1999). Mainly humoral reactions produce antimicrobial peptides to eradicate the pathogenic substances (Cociancich et al., 1994; Meister et al., 1997) and this is followed by a phagocytosis process and nodule formation reactions come, into force as immediate defense responses to infections (Miller et al., 1994). In addition cellular defense reactions have been reported (Jomori and Natori, 1992; Kawasaki et al., 1993; Charalambidis et al., 1996; Lanz-Mendoza et al., 1996; Foukas et al., 1998). However, compared to cellular reactions, humoral reactions play a vital role in immune defense. Insects seemingly lack any adaptive immune responses that operate analogously to the well documented antibody or histocompatibility adaptive immune responses in vertebrates (Hoffmann, 2003). In invertebrate immunity, LPS plays a role in the initial stage of signal transduction to activate acute phase protein genes. Future studies, especially in vertebrate and invertebrate immune surveillance and pathogen clearance, are likely to demonstrate the effectiveness of an innate immune system based on bacterial endotoxin.

Conflict of interest

None.

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