Bacterial Endotoxin Assays Relevant to Host Defense Peptides

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The Limulus amoebocyte lysate (LAL) test is the most sensitive and reliable assay for the detection of trace amounts of bacterial endotoxins (lipopolysaccharides, or LPS), and is an accepted in vitro alternative to the rabbit pyrogen test for evaluations of parenteral drugs, biological products, and medical devices. There are three principal LAL tests, which can be categorized as both semi-quantitative and quantitative methods, including gel-clot, turbidimetric, and chromogenic assays. Since the 1970s, these tests have been successfully formulated and commercialized by US and Japanese manufacturers. More recently, in addition to the recombinant factor C-based assay, a novel product containing all of the recombinant coagulation factors from horseshoe crab has been developed, which may lead to the creation of a next generation LAL alternative. Furthermore, there are antimicrobial peptides called "host defense peptides (HDPs)" that play a key role in innate immune responses. Thus, it is very important to properly evaluate the actions of HDPs (human defensins and cathelicidin peptide LL-37) such as the neutralization of LPS, immunostimulatory functions, and anti-endotoxin activity. Moreover, sensitive detection of LPS in cell culture media should be conducted to address the problem of endotoxin contamination in the media. Here, we discuss the progress of LAL-based endotoxin assay technologies, as well as their applications and limitations, with a focus on innovative functional studies of HDPs.

Key words: endotoxin, LPS, LAL test, endotoxin masking, host defense peptides

Introduction

Horseshoe crab (Limulus polyphemus) is often called a living fossil as it has been living on earth for more than 200 million years. In 1964, Levin and Bang discovered that Escherichia coli (E. coli) endotoxin and a marine Vibrio leads to the extracellular coagulation of Atlantic horseshoe crab blood. In the system, clotting enzyme cleaves a clottable protein (coagulogen) to produce coagulin, which forms an insoluble gel-like clot1). Thereafter they developed a sensitive assay for bacterial endotoxin using the blood cell lysate, Limulus amoebocyte lysate (LAL), which depends on the gel-clot formation in the presence of endotoxin a dominant type of pyrogen2). Bacterial endotoxins are biologically active compounds that compose the outer membrane of Gram-negative bacteria and its molecular weight is in the range of 100 kDa to 1,000 kDa showing both monomeric and aggregated forms in aqueous solutions because of their amphipathic properties. The chemical name, lipopolysaccharide (LPS) has been mostly used synonymously with bacterial endotoxin. LPS consists of two major...
parts, a hydrophobic domain known as lipid A and a hydrophilic polysaccharide portion. LPS also triggers monocytic cytokine production involved in inflammatory response, leading to systemic inflammatory diseases including sepsis and septic shock associated with high mortality. It became evident that endotoxins are the most ubiquitous pyrogens and those can be commonly found as a contaminant in pharmaceutical manufacturing. Based on these findings, the LAL endotoxin assay (LAL test) has been widely used as a successful alternative to the rabbit pyrogen test for pharmaceutical drugs and medical devices.

The rabbit pyrogen test is a qualitative in vivo assay to detect pyrogens by means of the intravenous injection of a sterile solution. Observing a change of body temperature in rabbits correlates with the presence of pyrogens. The test was widely adopted for many years for the quality control of parenteral drugs based on a positive correlation with the LAL test\(^3\). This method can detect endogenous pyrogens, such as interleukin-6 (IL-6), interleukin-1 (IL-1) and TNFα as well as bacterial endotoxins\(^4\).

In addition, antimicrobial peptides, also termed host defense peptides (HDPs) can play very important roles in innate immune responses. Several types of HDPs, including α-defensins (human neutrophil peptides, HNPs), β-defensins and LL-37, are known to be amphiphilic and mostly cationic polypeptides with diverse functions in innate immune system\(^5\). LL-37 is a member of HDPs (endotoxin neutralizing peptides) with the ability to bind to endotoxins, such as Lactoferricin-derived peptides, Magainin-2, Tachyplesin I, Polyphemusin I and LALF-derived peptides\(^6\). This review provides a comprehensive overview of the most recent insights regarding LAL-based endotoxin assay relevant to HDPs with a focus on LL-37 peptide.

**History of the LAL test**

In 1977, the Food and Drug Administration (FDA) approved the LAL test kit (gel-clot) that was first launched by Associates of Cape Cod, Inc. (ACC), MA, USA. In 1983, Seikagaku Corp. (Tokyo, Japan) developed an innovative LAL assay using a chromogenic substrate (Toxicolor), which is the most sensitive of the LAL assays. Then, ACC as well as Wako Pure Chemical Industries, Ltd. (Osaka, Japan), developed the kinetic turbidimetric LAL assay in combination with the purpose-designed instruments. These methods significantly improved the sensitivity and accuracy of the test. Nakamura et al.\(^7\) demonstrated that a cascade pathway for endotoxin-induced LAL coagulation consists of three sequential activations of hemolymph serine protease zymogens (endotoxin sensitive Factor C, Factor B and proclotting enzyme) (left side of Figure-1).

On the other hand, in the early 1980s, LAL was found to be also capable of reacting with (1→3)-β-D-glucan (beta-glucan), a major fungal cell wall component. It was thereafter shown that the

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**Figure 1** Scheme of two coagulation pathways in a horseshoe crab, T. tridentatus and the principle of the LAL test.
reaction occurs through activation of beta-glucan-sensitive coagulation factor (Factor G). Therefore, the conventional LAL test is not specific to endotoxin because of the presence of Factor G in the amebocyte lysate. Obayashi et al. has developed a new chromogenic endotoxin-specific assay by recombining the partially purified coagulation enzymes (serine protease zymogens). The recombined enzymes (Factor C, Factor B, and proclotting enzyme), and a chromogenic substrate, Boc-Leu-Gly-Arg-pNA reacted only with endotoxin to release p-nitroaniline (pNA). In addition, the enzymes did not react with beta-glucans. This new test (Endospecy, Seikagaku Corp.) has a definite advantage over the conventional LAL test with high specificity and a sensitivity of 1 pg/ml \((0.003\ \text{EU/ml})\) of E. coli 0111: B4 endotoxin. At the same time, Obayashi et al. also established a novel assay, G-test (Seikagaku Corp.) for beta-glucan specific quantification.

Recent progress in the LAL technology

A new recombinant LAL reagent (PyroSmart, Seikagaku Corp.) has been launched in December 2015 based on collaboration between Seikagaku Corp. and Kyushu University. The endotoxin determination kits utilizing recombinant Factor C (rFC) have already been commercialized by several companies, while PyroSmart is the first reagent kit having high sensitivity \((0.001\ \text{EU/ml in 30-min reaction})\) developed by recombining all of the recombinant coagulation factors from Asian horseshoe crab \((Tachypleus tridentatus)\) in the coagulation cascade. In the 1990s, Muta and Iwanaga successfully accomplished the cDNA cloning of horseshoe crab coagulation factors. Based on the findings, Ding et al. of National University of Singapore developed a novel assay (PyoGene) for endotoxin introduced by Cambrex (now Lonza) using rFC from Southeast Asian horseshoe crab \((Tachypleus rotundicauda)\). In recent years, two Germany companies, Hyglos and Haemochrom Diagnostica GmbH put the rFC-based endotoxin assay kits comparable to PyroGene \((0.005-5\ \text{EU/ml in 60-90 min reaction})\) on the market using different species of horseshoe crab.

In addition, rFC-based assay are introduced as valid alternative methods in the FDA “Guidance for Industry – Pyrogen and Endotoxins Testing: Questions and Answers”. However, such alternative procedures and methods should be validated to demonstrate the substantial equivalence to the bacterial endotoxin test (BET). We need to focus on the technical and regulatory trends of current topics on animal-free LAL alternatives.

LAL test methodology

The LAL test is far superior to any other endotoxin detection method, i.e. pyrogen test, immunological assays, and metachromatic assay, from the viewpoint of sensitivity, specificity, and accuracy. The LAL assay is based on the sequential activation of serine-proteases cascade in the amebocyte lysate. Endotoxins in the sample can activate the cascade system of endotoxin-mediated coagulation pathway (Factor C pathway). The gel-clot assay is a simple semi-quantitative method involving the cleavage of the clotting protein, coagulogen, and its conversion into insoluble coagulin polymers. The turbidimetric assays are photometric determination measuring increase in turbidity generation with end-point and kinetic procedures. Photometric assays utilize chromogenic assays in part; the cleavage of a chromogenic substrate is followed by the release of chromophore, pNA with the end-point and kinetic procedures. The yellow or purple color of the solutions following the diazo coupling step indicates the presence of endotoxin in the samples. More recently, Obata et al. introduced a new endotoxin assay platform based on laser light-scattering particle-counting methods to further increase sensitivity without any chromogenic substrates. Furthermore, the advanced technologies enabling automated robotic system have been introduced into the LAL market leading to cost saving and ensuring the reproducibility.

LAL test interferences

There has been much discussion of the significance and interpretation of BET. Interferences of the LAL test with samples remain a challenge to overcome, particularly in blood specimen. Interfering substances in test samples could cause a considerable inhibition or enhancement of any of the steps in the cascade, thus significantly affecting
the final results. Basically, LAL test is likely to be affected by many factors such as reaction temperature, pH, ionic strength, alkaline earth metal, chelating agents, proteins, nucleic acids, fatty acids, and surfactants. Interestingly, surfactants are capable to interact with endotoxins due to their amphiphilic character and resulting in altered supramolecular LPS structures. For example, Triton X-100, sodium deoxycholates, and sodium dodecylsulfate can affect the aggregation state and detection of endotoxin. In addition, beta-glucan is often problematic since the conventional LAL test reacts not only with endotoxins but also beta-glucans. This can be solved by replacing with endotoxin specific LAL tests. In addition, serine proteases, i.e. coagulation factor X, thrombin, plasmin, and trypsin, cause false-positive interference resulting from the cleavage of coagulogen or chromogenic substrate. On the contrary, serine protease inhibitors such as anti-thrombin, trypsin inhibitor, and plasmin inhibitor cause false-negative interference through a direct inhibition of coagulation enzymes. Although several methods have been used to remove or inactivate the interfering substances for the endotoxin-specific LAL tests using human plasma, serum and whole blood, important problems still remain unsolved to further improve the early detection of sepsis.

The presence of serum proteins, surfactants and chelating reagents changes the supramolecular structures of endotoxin aggregate, and thereby affects LAL test outcomes. The phenomenon of low endotoxin recovery (LER) was first reported by Genentech Inc. in April 2013. Certain formulations of biopharmaceuticals containing citrate, phosphate buffer, and polysorbate components that were spiked with a known level of endotoxin cannot activate LAL coagulation system, resulting in LER. It is noteworthy that the important feature of LER is time dependent decrease in endotoxin activity after spiked into the test sample. Thus, FDA has considered LER as a potential safety issue due to the possibility that products contaminated with undetected level of endotoxin by standard test methods may lead to undesired reactions in humans. To release masked endotoxin, extensive dialysis should be performed or, dispersing agents (Pyrosperse) as well as a variety of chaotropic and amphiphilic agents (Endo-RS) are often used before analysis.

### Pretreatment of samples to overcome interferences

Most samples including interfering substances can be applicable for the LAL test after dilution with endotoxin-free distilled water, although dilutions should not exceed the maximum valid dilution (MVD). The MVD is the maximum allowable dilution of a specimen at which the endotoxin limit can be determined. However, human and animal plasma, serum and whole blood cannot be subject to the LAL test by just dilution for the diagnostic purposes and the validation of sepsis models. In these cases, pretreatments of samples with chloroform, acetic acid, perchloric acid, nitric acid, potassium hydroxide and surfactants, or heating at high temperature have been reported to be more effective for endotoxin recovery. With regard to diagnostic purposes of the LAL test, an in vitro diagnostic (IVD) is already approved for septic patients by the Japanese Ministry of Health, Labour, and Welfare (MHLW); however, the kinetic turbidimetric assay after dilution and heating of the samples in the presence of a surfactant is now the only method, which can be used for IVD assay, although the sensitivity is very low. Thus, there are quite a few studies that aim to address the technical issues concerning the LAL-based endotoxin assays using blood samples. Interestingly, the method (EndoLISA; Pep–Abser) in which endotoxin molecules in the samples are first adsorbed to a solid phase coated with endotoxin binding protein/peptide for eliminating interfering substances and then endotoxin levels are analyzed, are expected to efficiently measure and evaluate the ultra–trace amounts of endotoxin in the blood.

### Interactions of endotoxins with proteins

It has been recognized that a number of proteins, e.g. LPS–binding protein (LBP), bactericidal/permeability-increasing protein (BPI), and several cationic proteins/peptides such as lysozyme and HDPs interact with endotoxin. Interestingly, LBP is involved in the immunological responses by interacting with endotoxin and signaling through endotoxin receptors, i.e. Toll–like receptor 4 (TLR4) and CD14. Moreover, much attention had been paid to the cationic HDPs from a new perspective in anti-infective therapeutic strategies. In this
context, structural analyses of HDPs in complex with endotoxins are of great importance for understanding the mechanisms related to the action of HDPs. However, there have been few well-conducted studies on the complex structures of HDPs–LPS or the structure of HDPs in the presence of endotoxin. Additionally, these interactions result in the masking of endotoxin molecules, and thus it is considerably difficult to completely remove the endotoxin from these binding compounds. Specific dissociation techniques using surfactants such as octyl-β-D-glucopyranoside and Triton X-100, and proteinases may efficiently improve the recovery of endotoxin from binding protein. However, it is not feasible at present to establish one general method for extracting endotoxin from all compounds.

Importance of endotoxin test for host defense peptides and its related samples

It has also been reported that HDPs provide an antibacterial, antifungal and antiviral function in addition to immunostimulatory activities including neutrophil chemotaxis and immunomodulatory effect. Nagaoka et al. demonstrated that human α-defensins, human β-defensins, and human cathelicidin LL–37 potently modulate neutrophil apoptosis. More interestingly, they recently discovered that LL–37 enhances the LPS uptake by endothelial cells. HDPs are also typically cationic peptides as a member of a family of endotoxin (LPS)-binding proteins. Basic methods to assess the LPS binding activity of HDPs include erythrocyte agglutination assay, dansyl polymyxin B displacement assay, analysis by reversed-phase HPLC, peptide-mediated inhibition of the binding of FITC-conjugated LPS/Alexa 488-labeled LPS to RAW 264.7 cells, peptide binding assay to LPS-coated microtiter plates, and Biacore kinetics assay. Furthermore, both anti-endotoxin and antibacterial activities can be explored by suppression of proinflammatory cytokine production by macrophage cell lines or macrophages, and antimicrobial actions in animal models. In order to properly evaluate the effect of these peptides on the neutralization of LPS and/or the immunomodulatory function in vitro and in vivo, it should be clearly demonstrated that a peptide in aqueous solution is endotoxin-free or being prepared for extremely low level of endotoxin using the LAL test. Moreover, it is very important to address the problem of endotoxin contamination in cell culture media used for in vitro studies of antimicrobial and anti-endotoxin activities. Kirikae et al. reported that considerable lots of fetal bovine serum (FBS) in 40 samples from 13 international manufacturers contain significant levels of endotoxin and beta-glucan, which must have a wide range of effects on the release of inflammatory cytokines into the culture medium.

Similarly, cationic proteins (such as lysozyme, ribonuclease A, and human IgG), made the detection of endotoxins with the LAL test even more difficult based on the formation of endotoxin–protein complexes due to their masking effects of endotoxin. Endotoxin neutralizing protein (ENP) is an anti-lipopolysaccharide (anti-LPS) factor derived from horseshoe crabs. ENP is bactericidal for gram-negative bacteria and is capable of neutralizing LPS. An ENP/LPS molar ratio of 20 : 1 completely inhibits the LAL assay. Consequently, it is a challenge to accurately determine the endotoxin levels in the presence of cationic peptides because of its ability to bind with endotoxin very tightly, and neutralizing the biological activity of endotoxin. In this case, highly effective methods to overcome masking, such as pretreatment with detergents, filtration, and protease digestion, will be needed. If such methods turned out to be ineffective, other approaches to adequately recover endotoxin need to be investigated. In general, according to the FDA guidance for industry, bacterial endotoxins assays are easily affected by the physical and chemical properties of the test samples. When such interferences cannot be reduced by sample dilution (up to the MVD) or other preparation methods, the rabbit pyrogen test should alternatively be used. If there is no possibility of being contaminated with non-endotoxin pyrogens, the rabbit pyrogen test can be feasible as an alternative approach for the LAL test. In fact, Kirikae et al. utilized the rabbit pyrogen test to examine endotoxin contamination in a LL37 derived peptide (hereinafter referred to as LL37P).

Anti-endotoxin activity of host defense peptides

To investigate the mechanism of how LL37P can
inhibit the endotoxin activity in vitro, Kirikae et al. looked at the effects of LL37P on LPS molecule itself or the LAL coagulation enzymes using the LAL test. The activity of the LAL coagulation enzymes consisting of Factor C and Factor G pathways cannot be inhibited by LL37P itself (Figure-2), and LL37P shows an inhibitory activity against the LAL test only by binding with LPS 41). In in vivo experiments, various animal studies were performed to investigate the protective effects of cationic peptides on the animal models of endotoxin-induced lethal sepsis 41)-44). In a rat septic shock model, giant silk moth-derived cecropin, which is the first HDP discovered, reduced the mortality, the bacterial count in the blood, and markedly lowered plasma endotoxin levels measured by conventional LAL test as well as TNF-α levels 43). Moreover, injection of LL37P successfully protected ceftazi-di-mine-treated, D-GalN-sensitized mice infected with P. aeruginosa from death, and markedly lowered plasma endotoxin levels measured by endotoxin-specific Endospecy test and serum TNF-α levels 41).

**Perspectives**

Gram-negative bacterial endotoxin (also called LPS), has been identified as a causative complicating factor in many serious infectious diseases. Polymyxins founded in 1947 are a group of cationic polypeptide antibiotics with strong bactericidal activity. After the discovery of polymyxins, a number of cationic peptides have been identified in a wide range of microorganisms, plants, and animals (invertebrates and vertebrates). Many studies have reported that these peptides possess inherent antibacterial and antiviral activities. Moreover, they have an amphipathic structure with hydrophobic and positively charged clusters. The structural properties of these peptides including synthetic derivatives are closely related to the binding affinity for LPS as well as their antimicrobial and antiviral activities 31)-45). HDPs are important molecules for the innate immune response and protect the host against pathogens. In mammalians, TLR4/MD2 and CD14 play a key role in LPS-triggered innate immune system leading to the production of proinflammatory cytokines. On the
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<th>Testing method</th>
<th>Specificity for endotoxin</th>
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contrary, in invertebrates, blood coagulation, melano-
ization via prophenoloxidase activation and induc-
tion of HDPs are the key players of the humoral immu-
ity. With regard to horseshoe crab, endotoxin–mediated coagulation cascade (Factor C pathway) and beta–glucan–mediated coagulation cascade (Factor G pathway) are regulated at an initial step of each cascade via the inhibition by anti-LPS factor and beta–glucan–binding protein, respectively, and both of these factors are involved in negative feedback regulation of exces-
sive blood clotting. Thus, these anti-LPS factor and beta–glucan–binding protein play an important role in cellular defense of horseshoe crab against microbial invasion.

The LAL test utilizing coagulation factors is the most sensitive and specific method to detect trace amounts of endotoxin in biological samples in both native and recombinant reagents. For HDPs, endotoxin detection kits, which are shown in Table-1 (native type B, recombinant type C/D), are available for endotoxin–specific assays. HDPs should be evaluated using endotoxin–free samples, or those prepared at extremely low level of endotoxin using the LAL test.

Finally the efficacy of cationic peptides for protection against endotoxin–induced lethality should be investigated in vivo using animal sepsis models. In these studies, a marked decrease in plasma endotoxin levels measured by the LAL test after treatment with HDPs clearly indicate how the peptides become effective in vivo. Moreover, the findings that LL37P does not show any significant inhibition against the endotoxin–mediated coagulation pathway in LAL, is considered extremely important for interpreting the endotoxin levels in experimental animal models using the LAL test. Thus, the decreased levels of plasma endotoxin result only from binding of LL37P to LPS.

Moreover, some of these peptides have been successfully modified by amino acid substitution, and replacement of natural amino acids with D-amino acids, with the aim of developing peptide derivatives with improved bactericidal activity, anti-endotoxin activities and proteolytic resistances. The modified peptides may have a potential to modulate apoptosis, enhance wound healing, and angiogenesis.

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