Review

The Molecular Mechanism of Interaction between Sushi Peptide and *Pseudomonas* Endotoxin

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Septic shock is caused by Gram-negative bacterial infection. Lipopolysaccharide (LPS) is the bioactive molecule present on the outer membrane of the Gram-negative bacteria. It is generally thought that LPS interacts with sensors on the host cell membrane to activate the intracellular signaling pathway resulting in the overproduction of cytokines such as TNF-α. This causes inflammation and ultimately, septic shock. Lipid A is the pharmacophore of the LPS molecule. Thus, developing bio-molecules which are capable of binding LPS at high affinity, especially to the lipid A moiety is an efficient way to neutralize the LPS toxicity. Factor C, a serine protease in the horseshoe crab ameobocytes, is sensitive to trace levels of LPS. We have derived Sushi peptides from the LPS-binding domains of Factor C. Our earlier study showed that the Sushi peptides inhibit LPS-induced septic shock in mice. Here, we demonstrate that the molecular interaction between LPS and Sushi 1 peptide is supported by the hydrophobic interaction between the lipid tail of LPS and Sushi 1 peptide. Furthermore, in the presence of LPS, the peptide transitions from a random structure into an α-helical conformation and it disrupts LPS aggregates, hence, neutralizing the LPS toxicity. *Cellular & Molecular Immunology*. 2006;3(1):21-28.

Key Words: septic shock, Sushi 1 peptide, Factor C, *Pseudomonas* endotoxin/LPS, LPS binding, electrostatic and hydrophobic interaction

Introduction

Gram-negative bacteria are the major pathogens responsible for a wide variety of infections and illnesses. During Gram-negative bacterial infection, the LPS, also known as endotoxin, causes excessive release of inflammatory cytokines, leading to multiple organ failure and death. The indomitable feature of LPS has been a major challenge to the pharmaceutical and medical industries. Thus, the development of a drug, which is effective in neutralizing endotoxin is urgently required. Efforts are currently underway in many labs worldwide to increase the potency and specificity of anti-LPS peptides so that they are specifically toxic to microbes but not to mammals. In our lab, we have developed Sushi 1 peptide (S1) from the LPS-sensitive protein, Factor C. We have proven by a series of approaches, that S1 peptide can effectively bind LPS and neutralize its endotoxicity.

Septic shock

Pyrogens are substances that cause fever when introduced intravenously. The best studied pyrogen is LPS found in the outer membrane of Gram-negative bacteria. During Gram-negative sepsis, LPS stimulates host macrophages to release inflammatory cytokines, causing inflammation which informs the host of pathogen invasion. However, excessive inflammation causes septic shock, multiple organ failure and death. In the United States, a multicentre observational cohort study has projected an estimated 751,000 cases of sepsis per annum (1).

LPS structure

Studies on the molecular biology of septic shock have so far focused on the chemical structure of LPS. Generally, LPS has a tripartite structure comprising three covalently linked domains: the O-specific antigen, the core oligosaccharide and lipid A (Figure 1). (a) The O-specific antigen is made up of a

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Abbreviations: CD, circular dichroism; LBP, LPS-binding protein; LPS, lipopolysaccharide; S1, Sushi 1 peptide; τ_D the diffusion time; τ_{DL}, the diffusion time of the large particle.

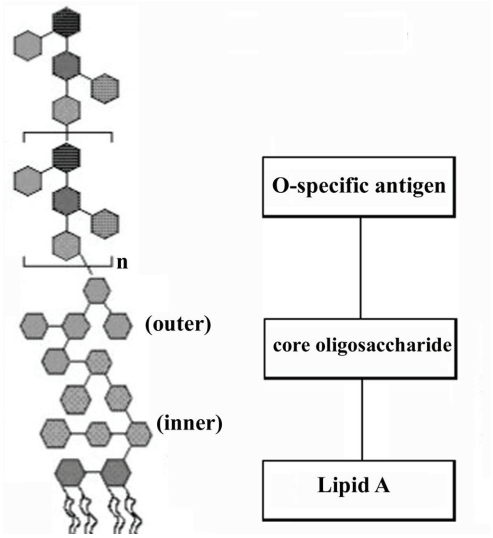

Figure 1. The chemical structure of lipopolysaccharide. LPS consists of an O-specific antigen, a core oligosaccharide and a fatty acid tail called lipid A. The core oligosaccharide, which varies from one bacterial species to another, is made up of outer and inner sugar regions. Lipid A virtually always includes two glucosamine residues modified by phosphates and a variable number of fatty acid chains. Adapted from Ulmer (57), with some modifications.

chain of repeating oligosaccharides of 3-8 units, which are strain-specific and determine the serological identity of the respective bacterium (2). Therefore, considerable structural diversity is noted amongst the O-antigen chain structures of LPS from various Gram-negative bacteria. Furthermore, the major anti-LPS immune response is generally directed to the O-chain polysaccharides. However, the O-specific antigen of LPS is not necessary for bacterial growth or survival (3). (b) The core oligosaccharide has an inner KDO (2-keto-3-deoxy-D-mano-octonate)-heptose region and an outer hexose region, primarily glucose, galactose and GlcNAc (4, 5). The hexose molecules in the outer core are more variable in structure than the inner core. The KDO sugar is linked directly to the lipid A moiety (6). (c) The minimum structure of LPS capable of sustaining bacterial growth and survival consists of the lipid A attached to two or three KDO residues (7). Lipid A is the most conserved moiety of LPS derived from diverse strains of Gram-negative bacteria. It is considered to be the bioactive centre of the LPS molecule as virtually all LPS-induced biological responses are lipid A-dependent. The most convincing evidence comes from studies of free lipid A (8) and synthetic lipid A (9), both of which show full endotoxic activity. Lipid A is composed of a phosphorylated β1,6-linked D-glucosamine disaccharide that carries variable numbers of asymmetrically placed amide or ester-linked acyl chains. This structure is the minimal requirement for lipid A bioactivity, referring to its cytokine inducing capacity (6). The unique structure of lipid A most likely reflects its important roles in the outer membrane assembly and functions, and it ensures resistance to phospholipases. Differentiation in phosphorylation or acylation patterns of the hexosamine disaccharide can affect the endotoxicity of the LPS molecule.

Gram-negative bacterial infection and LPS signaling pathway

During infection, the host innate immune defense immediately responds to the pathogen invasion *via* LPS-induced signal transduction pathways, finally resulting in inflammation and septic shock. The innate immune system initiates host defense against the invasive microbial pathogens *via* specific recognition mechanisms. LPS is a pathogen-associated molecular pattern of the Gram-negative bacteria and it is a key virulence factor. Upon infection, LPS is released from the bacterial cell wall into the blood stream and the LPS-binding protein (LBP) binds to LPS *via* the lipid A moiety (10). The LBP-LPS complex subsequently interacts with CD14 (11), a 55 kD glycophosphatidylinositol (GPI)-linked receptor protein that is found on the surface of macrophages, monocytes and neutrophils (12, 13).

Another LPS sensor molecule is the Toll-like receptor 4 (TLR4)-MD-2 complex, which is localized to the host immune cell membrane (14). LPS-induced signal transduction involves many protein kinases, such as p38 and JNK (15), which are necessary for the activation of several transcription factors such as NF-κB, which in turn activates gene transcription of numerous pro-inflammatory cytokines, tissue factor, adhesion molecules and inducible nitric oxide synthetase. Subsequent overproduction of these potent mediators initiates a series of events that lead to clinical manifestations of sepsis. Amongst the pro-inflammatory cytokines, TNF-α plays a critical role in the inflammatory response and is often regarded as a hallmark of LPS-induced inflammatory study (16). Following LPS challenge, the immunological cascade, encompassing CD14, TLR, MAPK and NF-κB (17) is swiftly activated to sensitize the host to an LPS-induced uncontrolled acute inflammatory response that results in septic shock (Figure 2). However, at the initial step, the binding of the LBP to LPS is required to trigger an inflammatory response. Thus, our study is directed towards the intervention of this step with potential LPS-binding drugs to compete against LBP binding and to attenuate the damage to the host.

Current advances on LPS-neutralizing strategies

In light of the role played by LPS in septic shock, it is the most prominent target for prophylaxis. This paper explores the development of LPS antagonists. Blocking sepsis at the point of LPS itself is an approach that is highly advantageous because it is the most upstream step and the activation of the downstream inflammatory cascade may be checked or prevented. Current anti-LPS strategies under investigation include LPS analogues, anti-LPS antibodies, and LPS neutralizing proteins or peptides.

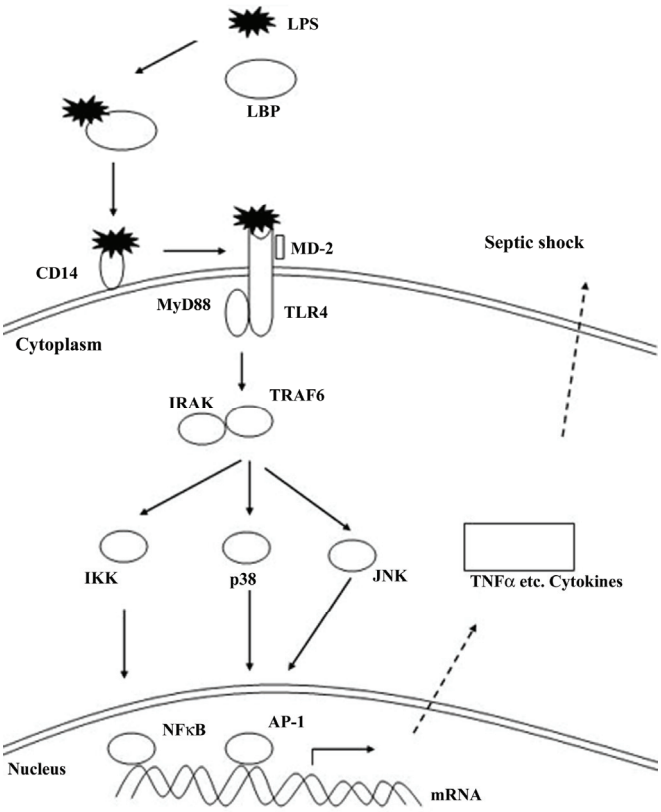

Figure 2. LPS-stimulated signaling pathways in a host. LPS binds to the host serum protein, LBP, and the LPS-LBP complex is transferred to the receptor, CD14, at the cell surface. LPS then interacts with the signaling receptor TLR4, with the adapter protein MD-2. LPS stimulates the activation of various signaling pathways, including the IKK-NFκB, p38, and JNK pathways. Furthermore, LPS activates the host immune gene expression *via* translocation of NF-κB and AP-1. Subsequently, the cytokine genes are expressed. Overproduction of TNF-α and the ILs will lead to septic shock. Adapted from Guha and Mackman (17) with modifications.

Investigations into LPS analogues have focused on the biologically active part, lipid A. These analogues should maintain the basic features of LPS, or those that competitively bind with host LPS-effectors against LPS without activating the host immune response. The first analogue that was discovered was lipid X, the mono-saccharide lipid A precursor, which inhibits the LPS-induced superoxide production in an *in vivo* neutrophil system (9, 18). So far, there are several kinds of LPS analogues reported, including E5331 derived from *Rhodobacter capsulatus* LPS (19), monophosphoryl lipid A (20) and *Helicobacter pylori* LPS (21). However, most of these compounds have limited LPS-inhibitory effects and some analogues could still induce some inflammatory activation. Anti-LPS antibodies could be raised to target different parts of the LPS. The first anti-LPS antibodies used in clinical trials were polyclonal human antisera obtained after vaccination of volunteers with heat-killed *E. coli* 0111-mutant J5 (22). The problems of polyclonal antibody preparation and difficulties of getting adequate supply of human antisera, led to the development of monoclonal antibodies against lipid A (23, 24). The application of some antibodies against the O-specific antigen is largely limited by the fact that the O-specific antigen is antigenically distinct for each strain or serotype of bacterial LPS. It has thus not been possible to create an antibody against the whole range of LPS molecules from strains that elicit ill effects. Furthermore, some antibodies like anti-*Pseudomonas* LPS have never shown any potential for protective efficacy (25). In light of difficulties and lack of efficacy of the anti-LPS antibodies, other approaches were sought.

LPS-binding peptides with antimicrobial properties

Numerous proteins and peptides in insects and mammals appear to have evolved for the purpose of binding and neutralizing LPS. For example, the bacterial permeability inhibiting factor, which binds LPS, has been extensively studied and is currently undergoing clinical trials. It has been reported that the bacterial permeability inhibiting factor could displace LPS from LBP-LPS complex by binding to the lipid A moiety (26) and this resulted in the inhibition of LPS-mediated proinflammatory effects *in vivo*. Recently, many efforts have been made to derive the LPS-neutralizing peptides from these proteins, since peptides are less immunogenic, readily produced by chemical synthesis and their structures are more easily determined. Thus, LPS-neutralizing peptides have been derived from the naturally occurring proteins such as cationic antimicrobial protein, LBP, serum amyloid P component, and Factor C (27-31).

The sensitivity of the horseshoe crab Factor C to LPS

The evolutionary success of horseshoe crabs over 500 million years attests to its strong immune defense mechanisms. It has relied solely on innate immune defense to thrive in microbiologically challenging habitats, where myriads of pathogens are found. The horseshoe crab uses several frontline defense mechanisms to stave off an infection: (a) complement cascade (32); (b) melanization reaction in a phenoloxidase-induced production of toxic compounds (33); (c) an extremely sensitive blood coagulation cascade that is triggered by trace levels of LPS (34, 35); (d) antimicrobial peptide defense (36, 37).

The horseshoe crab hemolymph contains mainly one type of cells called amoebocytes, which are extremely sensitive to LPS. The amoebocytes release granular components into the plasma to participate in self-defense *via* blood coagulation (38) and incapacitation of the invading microbe. The amoebocytes contain two kinds of secretory granules, the large and small granules. Studies on these granules suggest

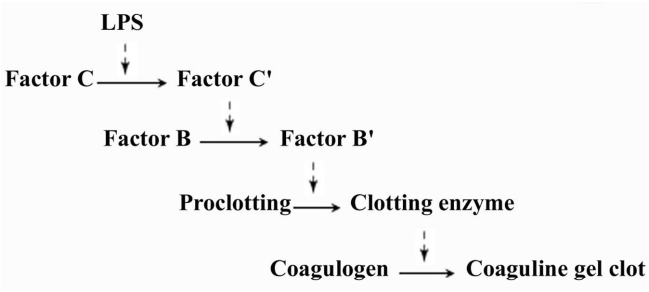

Figure 3. The coagulation cascade reaction in the horseshoe crab amoebocyte lysate. By a series of limited proteolysis, LPS activates proenzyme Factor C to become active Factor C', which in turn activates the proenzyme Factor B into Factor B'. Then proclotting enzyme is converted to clotting enzyme which proteolyses coagulogen into coagulin gel clot. This cascade of reactions has formed the basis of a very powerful diagnostic for LPS, a test that is FDA approved for quality assurance of pharmaceuticals and medical devices for human use. Adapted from Iwanaga et al. and Ding & Ho (39, 41).

that coagulation factors, such as Factor C, are localized in the large granules and that the antimicrobial peptides such as tachyplesin, are located exclusively in the small granules (35). In the past decade, the molecular mechanisms of the coagulation cascade (Figure 3) have been established (34, 39, 40). Factor C functions as a biosensor of LPS at the initial step of the coagulation cascade. Since Factor C can be activated by picogram levels of LPS (41), it is conceivable that it harbours LPS binding motif(s) that exhibits exceptionally high affinity for LPS (31, 42). Thus, it is logical to derive LPS binding peptides from Factor C.

The rationale for the derivation of LPS-binding sushi peptides from Factor C

In our lab, we have characterized the LPS binding region of Factor C in the species of horseshoe crab, *Carcinoscorpius rotundicauda*. The Factor C (CrFC) is a 132 kDa glycoprotein, consisting of a heavy chain (80 kDa) and a light chain (52 kDa). Near the N-terminus of the heavy chain, there are several repeating units of sushi domains of ~ 60 amino acids each. Expression and analysis of the N- and C-terminal fragments of recombinant Factor C protein revealed the major LPS-binding site to be located to the N-terminal region (43). The N-terminal fragment (CrFCES) was firstly subcloned and expressed, and proven to bind LPS with high affinity (42). Subsequently, small fragments in CrFCES were further subcloned and expressed showing that the Sushi 1 and Sushi 3 domains (Figure 4) are the major LPS-binding regions (31, 44). Although the LPS-binding sites of Factor C have been discovered, it was necessary to define the precise amino acid residues in the Sushi domains that are responsible for interacting with LPS. Based on our understanding of the amino acid sequence in the sushi domains and comparison of LPS-binding motifs of several other LPS-binding proteins, we showed that a predominance of lysine and arginine residues frequently occurs in alternation with hydrophobic residues (45, 46). Thus, a 34-amino-acid sequence was found within the Sushi 1 domain. The core LPS binding region of Sushi 1 resides in this 34-mer peptide, henceforth referred to as S1.

S1 peptide inhibits LPS-induced cytokine release in the mammalian host cell

We have shown that the S1 peptide could rescue mice from LPS-mediated lethality (Figure 5). Preincubation of 75 μg S1 with LPS for 30 min prior to intraperitoneal injection conferred 20-55% protection against LPS-induced lethality (31). This indicates a beneficial intervention on parameters that determine long-term survival and may provide a window of time for other therapeutic support. The possibility of a peptide being able to protect against the severe clinical symptoms of LPS-induced septic shock is a promising development. We have also shown that S1 inhibits TNF-α production by human macrophage, THP-1 cells (31, 42).

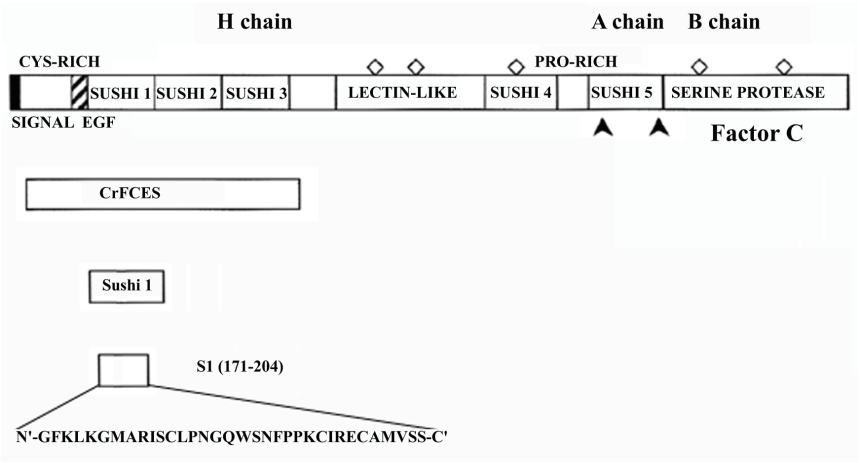

Figure 4. Factor C and its derived Sushi peptide, S1. Factor C is a multidomain protein with mosaic structure. The N-terminal domains of CrFCES contain the LPS-binding region. Further studies showed that Sushi 1 is one of LPS-binding regions and a 34-mer S1 peptide derived from Sushi 1 domain is the key LPS binding sequence. The relative positions of the truncated fragments of the Factor C protein are illustrated as open boxes. Adapted from Tan et al. (31).

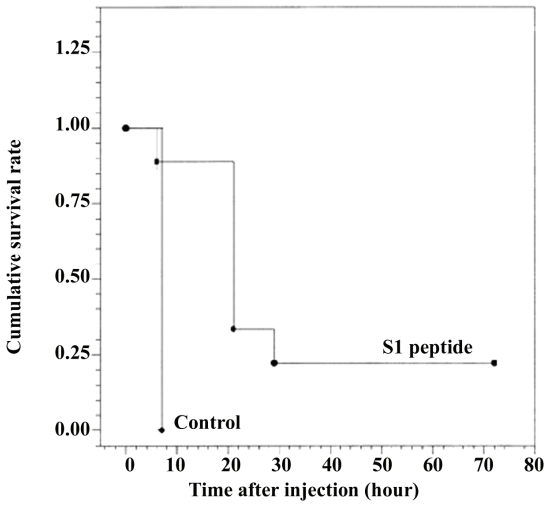

Figure 5. S1 peptide protects galactosamine-sensitized C57BL/6J mice against LPS-induced septic shock. Using 2 ng of *E. coli* 055:B5, a 100% LPS-induced lethality was achieved within 7 h. When LPS was preincubated with 75 μg of S1 peptide, increased the percentage of survival by 22%. Adapted from Tan et al. (31).

The molecular mechanism of action of S1 peptide in neutralizing LPS

Realtime biointeraction between S1 and Pseudomonas LPS

To clarify how S1 peptide can exhibit such strong antagonistic effect on LPS, we studied the molecular interaction between S1 peptide and LPS in the current work. Figure 6 shows a binding profile of S1 peptide to *P. aeruginosa* LPS. Our efforts have been focused on *Pseudomonas* as this is a recalcitrant Gram-negative bacterium that has acquired multiple drug resistance and is posing a serious threat to human health and the biomedical industry (47). We coated the *Pseudomonas* LPS on a biacore HPA chip activated with a hydrophobic surface. Unique biphasic association and dissociation profiles were observed when S1 peptide was flown over the microfluidic surface of the biacore chip, suggesting the binding of S1 to the immobilized LPS. Such realtime biointeraction analysis measured as a surface plasmon resonance, of the binding between S1 and LPS was carried out. A set of sensorgrams showing the profiles of interaction between different concentrations of S1 and LPS were used to demonstrate the affinity of S1 for the *Pseudomonas* LPS. The increasing association curves describe the initial binding of the peptide to the LPS immobilized on the HPA chip, and the decreasing curves relate to the dissociation at real time. The sensorgrams revealed that the signal intensity measured in response unit (RU) increased as a function of the concentration of the peptide bound to the immobilized LPS. This indicates that the amount of peptide bound to the LPS is related to the affinity of the peptide for the chip.

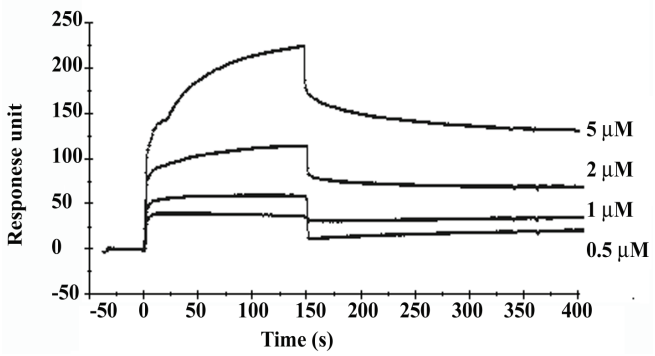

Figure 6. A sensorgram depicting the interaction of S1 with immobilized *P. aeruginosa* LPS. Aliquots of 100 μl each of four concentrations (0.5, 1, 2, 5 μM) of S1 peptide were injected over an HPA chip which was immobilized with *Pseudomonas* LPS. The S1 peptide interacted with the LPS and resulted in a sustained increase in the relative response unit. During the dissociation phase, pyrogen-free buffer was flown through the microfluidic surface at 20 μl/min. The surface was regenerated by a pulse of 100 mM NaOH prior to each injection.

The biophysical interaction between S1 peptide and Pseudomonas LPS

We used Trp fluorescence scanning spectroscopy to study the hydrophobic interaction between S1 peptide and *Pseudomonas aeruginosa* LPS. The penetration of S1 into the LPS aggregates was confirmed by determining the fluorescence emission spectra due to the Trp residue in S1 when in the presence of the LPS. The addition of LPS to the peptide sample caused a blue shift in the emission peak, from 354 nm to 350 nm (Figure 7A). This blue shift indicates that the Trp residue was partitioned into a more hydrophobic environment. The fluorescence intensity increased, suggesting that the Trp residue was more sterically confined. The Trp fluorescence scanning spectroscopy experiment revealed that the microenvironment of the S1 peptide was transformed from a hydrophilic to a more hydrophobic state (48, 49), particularly that of the anionic LPS. It is consistent with other studies on small peptides with Trp residue, using NMR, Raman and fluorescence measurements, indicating that initially, the peptides bind parallel to the acyl chains of lipid layer (50, 51). Based on the LPS structure, only lipid A could offer a more hydrophobic environment. It suggests that the Trp residue of S1 peptide was located in the hydrophobic acyl chains of lipid A in LPS. Because lipid A is the bioactive part of LPS, the binding of S1 peptide with lipid A could decrease the interaction of LPS with other LPS binding sensors in the host, such as LBP.

A conformational change could occur to the peptides because of their hydrophobic interaction with the LPS aggregates. Therefore, we used CD measurements to examine the secondary structure of S1 peptide. In aqueous solution, S1 adopts a random coil conformation as shown by the single minimum at 200 nm (Figure 7B). In the presence of LPS, a conformational change in S1 was observed. The

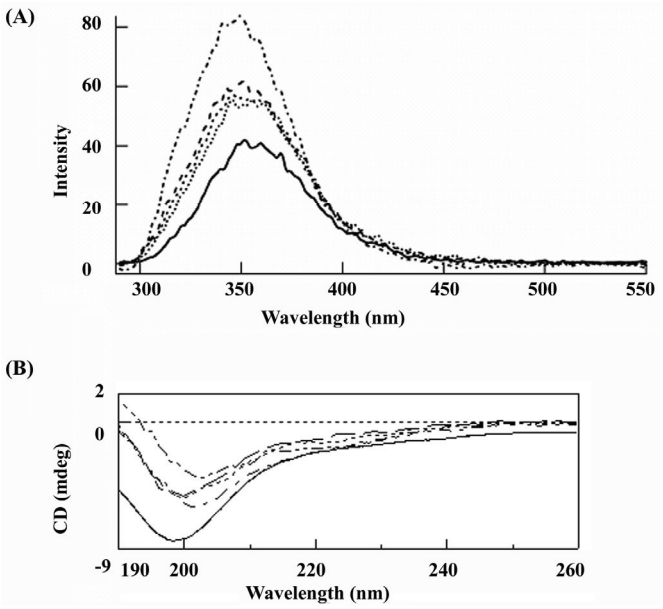

Figure 7. Hydrophobic interaction between S1 and *Pseudomonas* LPS. (A) Trp fluorescence scanning spectroscopy shows the excitation fluorescence spectrum of S1 in different concentrations of *P. aeruginosa* LPS. (B) CD spectra of S1 peptides in the presence of *P. aeruginosa* LPS. The samples were all dissolved in 10 mM phosphate buffer, pH 7.2. The changes in the spectra of the S1 peptide in 0 μM LPS (——); 10 μM LPS (- - - -); 20 μM LPS (·····); 50 μM LPS (- · - · -) and 100 μM LPS (— · · —) were recorded.

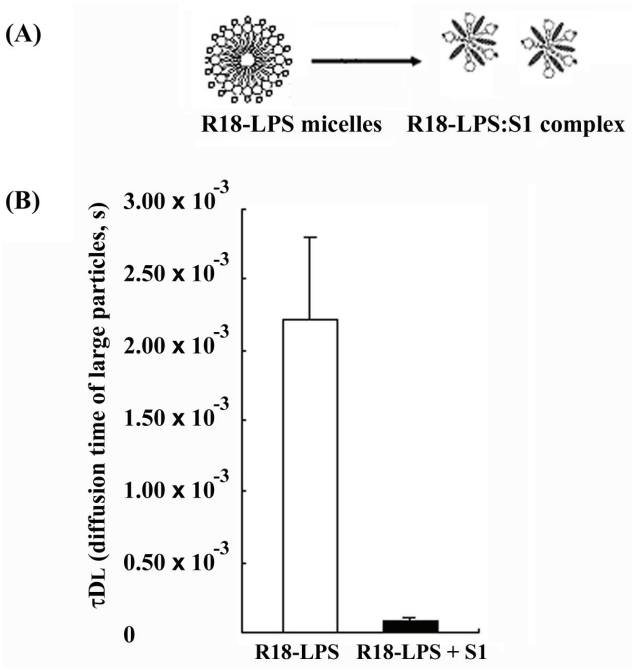

Figure 8. Determination of the stoichiometry of interaction between R18 labeled *Pseudomonas* LPS micelles and S1 peptide. (A) Interaction with increasing concentrations of S1 peptide caused a decrease in the diffusion time (τ_{DL}) of the complex as the large R18-LPS micelle was disrupted into small S1:R18-LPS micelles. (B) The concentration of fluorescent particles was measured for samples of R18-LPS under different conditions. The R18-LPS was held at a constant concentration of 100 nM while the S1 peptide was at 10 μM. All data points were averaged over 10 experiments.

spectrum suggests that S1 forms an α-helix. Hydrophobic interaction contributes significantly to the binding between the S1 and bacterial LPS (45, 46, 52). LPS forms micelles or vesicles in solution (2), with the bioactive part, lipid A, facing inside. The compact α-helical structure of S1 peptide enables S1 to insert into the LPS micelles or vesicles in solution and capture the lipid A moiety. Thus, the present study shows that the hydrophobic interaction between S1 peptides and the lipid A moiety of LPS is indispensable.

S1 peptide breaks LPS aggregates

Generally, LPS exists in the form of LPS aggregates/ micelles, which are governed by non-polar interactions between neighbouring alkyl chains as well as by bridges generated amongst phosphate groups by bivalent cations (2). Thus, LPS micelles are much more stable and may even be found in purportedly 'ultrapure' water. The observation of the different forms of LPS in solution has prompted us to investigate the change in LPS micelles or vesicles in the presence of S1. To this end, we used fluorescence correlation spectroscopy. *Pseudomonas aeruginosa* LPS labeled by R18 (53) was used in fluorescence correlation spectroscopy experiments (54). The background control showed no interaction between R18 and S1 at the concentrations used. The small component is free R18-LPS (including R18-LPS: S1 complex), while the large component represents R18-LPS micelles (including R18-LPS micelle: S1 complex) (Figure 8A). In the absence of S1, large fluorescent micelles of R18-LPS were present. However, the diffusion time, τ_{DL}, (diffusion time of large particle) was dramatically decreased in the presence of 10 μM of S1 peptide, indicating that the S1 interacts with and perturbs the large LPS micelles. LPS monomers have to be explicitly created by using detergents, such as the Triton series, which can be employed in two-phase endotoxin extraction (55). Triton X-100 was shown to interact with LPS micelles (54). Like Triton X-100, the S1 peptide interacted with R18-LPS to decrease its τ_{DL} and exhibited the same specific activities for LPS micelles (Figure 8B). Thus, S1 peptide was shown to attain a "detergent-like" activity. This is consistent with the report by Bechinger (50). It is possible that during interaction, S1 binds the acyl chain of lipid A, thus disrupting the critical force, which maintains the stability of the LPS micelles. The Gram-negative bacteria display and release a variety of different LPS formats from their outer membranes. It has been recently reported that the aggregated form of LPS is biologically more active than the free LPS molecules in the presence of LBP or of human serum (56). Here we have shown that the LPS aggregates were disrupted by S1, which may be related to LPS neutralization.

Future perspectives in the development of LPS antagonists

Increasing incidence of antibiotic resistant bacteria has compelled the urgent need for an alternative to traditional antibiotics. Since LPS has been found to interact with some LPS effectors or receptors on the host cell membrane, deriving some small molecule drugs which bind LPS at high affinity and neutralizing its toxicity is an efficient approach to the development of alternative frontline antibiotic against Gram-negative bacteria. This is especially so since LPS is conserved as an invariant essential chemical component present on the outer membrane of Gram-negative bacteria, acting as an important shield for their survival. To fight against bacterial infection, nature has endowed in almost all forms of life, an effective innate immune system, of which some sensor proteins play the key role.

Even though S1 peptide is a potential LPS-antagonist, various considerations should be noted that (1) in addition to its structure, the maintenance of peptide solubility is a critical parameter in the design of an improved LPS-binding and neutralizing peptide; (2) the peptide mimic should be nontoxic to humans; (3) the peptide should be resistant to degradation by proteases *in vivo*, especially prior to reaching its site of antimicrobial action, thus a prolonged half-life of the peptide would be desirable.

Acknowledgements

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