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Monomeric lipopolysaccharide (LPS) activity affected by dimerization of receptors is the key for the similarity between the *Limulus* amebocyte lysate (LAL) test and the rabbit pyrogen test

Masakazu Tsuchiya [1,*], Yayoi Miyagawa [2], Kohshi Kikuchi [3] and Shuji Adachi [2]

[1] *Microbial Solutions, Charles River, 1023 Wappoo Road, Suite 43B, Charleston, SC 29407, USA.*
[2] *Faculty of Bioenvironmental Sciences, Kyoto University of Advanced Science, 1-1 Nanjo-Ohtani, Sogabe-cho, Kameoka, Kyoto 621-8555, Japan.*
[3] *Faculty of Liberal Arts, National Institute of Technology Kumamoto College, 2659-2 Suya, Koshi, Kumamoto 861-1102, Japan.*

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Abstract

Endotoxin or lipopolysaccharide (LPS) triggers inflammatory cytokine production of mammal cells through Toll-like receptor 4 (TLR4) activation. This reaction causes pyrogenicity in rabbits. The rabbit pyrogen test has been used for the pyrogen detection in parenteral drugs and medical devices. *Limulus* amebocyte lysate (LAL) test, adopted as an alternative test for the rabbit pyrogen test in pharmacopoeias, has been safely used for more than 40 years. Even though the mechanisms of these biological assays are different, there should be similarity between the LAL test and the rabbit pyrogen test. Biological activity relates to the aggregation states of LPS, and there is still controversy whether the biologically active form of LPS is monomers or aggregates. Previous studies showed inconsistent results in biological activities of monomeric LPS. This suggests the difficulty to prepare monomeric LPS. Therefore, a simulation can be helpful to understand the activity of different status of LPS. In this study, the TLR4 activation was simulated by using simplified models, and compared the activity of LPS in different forms. The activation of TLR4 and the LAL activation require dimerization of receptors, which reduces the activity of monomeric LPS. The inert monomeric LPS caused by the dimerization of the receptors is the key to the similarity between the LAL test and the rabbit pyrogen test.

Keywords: Endotoxin; Lipopolysaccharide; Toll-like receptor 4; Innate immunity; *Limulus* amebocyte lysate: Bacterial Endotoxins Test

1. Introduction

Endotoxin is lipopolysaccharide (LPS) which is the major cell wall component of the outer membrane of Gram-negative bacteria. It is recognized as a pathogen-associated molecular pattern [1], and demonstrates multiple biological activities, such as pyrogenicity [2]. Since LPS is ubiquitous in the environment and is one of the most potent natural pyrogens, parenteral drugs and implanted medical devices are required to be tested for endotoxin contamination by the Bacterial Endotoxins Test (BET) using *Limulus* amebocyte lysate (LAL) derived from horseshoe crab blood cells [3]. The BET was adopted as an alternative method for the rabbit pyrogen test after positive correlation was observed between the LAL test and the rabbit pyrogen test in intensive studies by regulatory authorities and industry [4]. The principle of the activation of the LAL is a sequential activation of zymogens triggered by endotoxin [5]. The mechanism of the pyrogenic reaction in rabbits by LPS is due to cytokine production by the Toll-like Receptor (TLR) 4 activation that is totally different from the activation of the LAL [6]. Despite the difference, the BET, as the alternative test for the rabbit

* Corresponding author: Masakazu Tsuchiya
Microbial Solutions, Charles River, 1023 Wappoo Road, Suite 43B, Charleston, SC 29407, USA.

pyrogen test, has been safely used to detect LPS contamination in the products for more than 40 years. Therefore, there should be similarity in mechanism of activity control between the LAL test and the pyrogenic reaction in animals.

The mechanism of the activation of Factor C, the first zymogen in the LAL, has been elucidated that dimers or multimers of Factor C molecules are activated on LPS aggregates by intermolecular autocatalytic reaction [7]. Given the mechanism of the LAL activation, LPS aggregates, not LPS monomers, are active units in the LAL test.

Recent studies have revealed that the final stage of the mechanism by the innate immune system is triggered by LPS. Monomeric LPS is transferred from LPS aggregates to a complex of TLR4 and myeloid differentiation factor 2 (MD2) [8]. Dimerization of the resulting TLR4-MD2-LPS complex triggers TLR4 activation to produce inflammatory cytokines [9,10]. The transfer of LPS monomers from LPS aggregates to TLR4-MD2 complexes requires sequential steps that involve LPS binding protein (LBP), CD14, and serum albumin [11]. LBP binds LPS aggregates and transfers an LPS monomer to CD14 [8]. CD14 transfers the monomeric LPS to the TLR4-MD2 complexes on the host cell. These findings clearly indicate that the activation of the innate immune system is caused by monomeric LPS at the final stage of the TLR4 activation. However, there are still controversial discussions as to whether the biologically active units are LPS monomers or aggregates [12-16]. This is a macroscopic question on the biological activity of LPS, and there is not a definitive conclusion yet. Ribi et al. [12] reported that the disaggregated LPS to the monomeric size by deoxycholate did not show pyrogenicity, and removal of deoxycholate recovered the aggregation and pyrogenicity of LPS. Takayama et al. [13,14] observed cell activation with their monomeric LPS preparations, and Mueller et al. [15] did not. These inconsistent results suggest that there are experimental difficulties. LPS is amphiphilic substance with several fatty acids, and tends to be aggregated in water. It is probably difficult to maintain monomeric LPS in water, even if the concentration is below the critical micelle concentration.

Above considerations suggest that the aggregation status of LPS and the activity of monomeric LPS can be important factors for the similarity between the LAL test and the TLR4 activation. If the preparation of monomeric LPS is difficult, a simulation model will be useful to evaluate the effectiveness of LPS aggregates and monomers to show the biological activities. The purpose of this study is to find the key factors for the similarity between the LAL test and the TLR4 activation using a simulation of simplified TRL4 activation model that can distinguish between monomeric and aggregated LPS.

2. Model description

A cell surface was regarded as a square for mathematical simplicity. One side of the square was divided into 10^2 to consist of 10^4 lattices. Each lattice was numbered from 1 to 10^4 from the left end of the first row to the right end of the 100th row. The probability of TLR4 existing on the cell surface was assumed to be 0.01, 0.02, 0.05, 0.1, and 0.2. A random number of 0 to 1 was generated times equal to or half the number of TLR4 and was multiplied by 10^4 to determine the lattice where TLR4 exists. When a TLR4 was assigned to the lattice where TLR4 had already existed, a random number was generated again, and all TLR4s were set to be in any lattice.

Two models were considered for existence of TLR4. In model 1, TLR4 locates randomly in the lattices on the cell (Figure 1 (a)), and in model 2, two molecules of TLR4 are present in pairs (Figure 1 (b)). Figure 1 gives an explanation of the calculation method, the numbers of grids and TLR4 are set to be $10 \times 10 = 10^2$ and 20, respectively, for easy understanding. In model 2, two molecules of TLR4 exist in the lattice determined by generating random numbers and in its right-hand neighboring lattice (labeled A and B in Figure 1 (b)). When TLR4 (A or B) is assigned to the lattice where a TLR4 has already been placed, random number generation is performed again to determine the position of A. When the position of B is already occupied by a TLR4, no re-location was performed.

The total number of LPS, C, was assumed to be 125, 343, 729, 10^3, 3×10^3 or 10^4, and the LPS molecules form cubic aggregates composed of m LPS molecules on one side. The number of LPS aggregates is Round (C/m^3), where Round means integerization. A random number was generated to determine the lattice to which an aggregate binds. A square face with m^2 lattices contacts the cell surface. The lattice was the upper left corner of the square consisting of m^2 LPS molecules. The part where LPS aggregates spread outside the right or lower frame of the cell surface (lattice) was ignored. A random number was generated Round (C/m^3) times on the cell surface to determine the lattice to which the aggregate binds. When the lattice was already occupied by LPS, the LPS is considered to be stacked or overlapped.

Two cases are considered for TLR4 activation. In case A (Figure 1 (a1) and (b1)), all the TLR4 (darkly shaded) in contact with LPS aggregates (shown as a thick square for the case of $m = 3$) are assumed to be activated in both models 1 and 2. In case B (Figure 1 (a2) and (b2)), only two molecules of TLR4 (darkly shaded), which are adjacent to each other in the

region covered by the LPS aggregates, are activated to transmit a signal in both models 1 and 2. The number of activated TLR4 was enumerated by dividing twice the number of darkly shaded pairs by the total number of TLR4.

Attempts to determine the number of activated TLR4 were 50 times when C = 125, 343, 729, 10^3, 3×10^3, and 10 times when $C = 10^4$ in both models 1 and 2, and the mean and standard deviation were calculated.

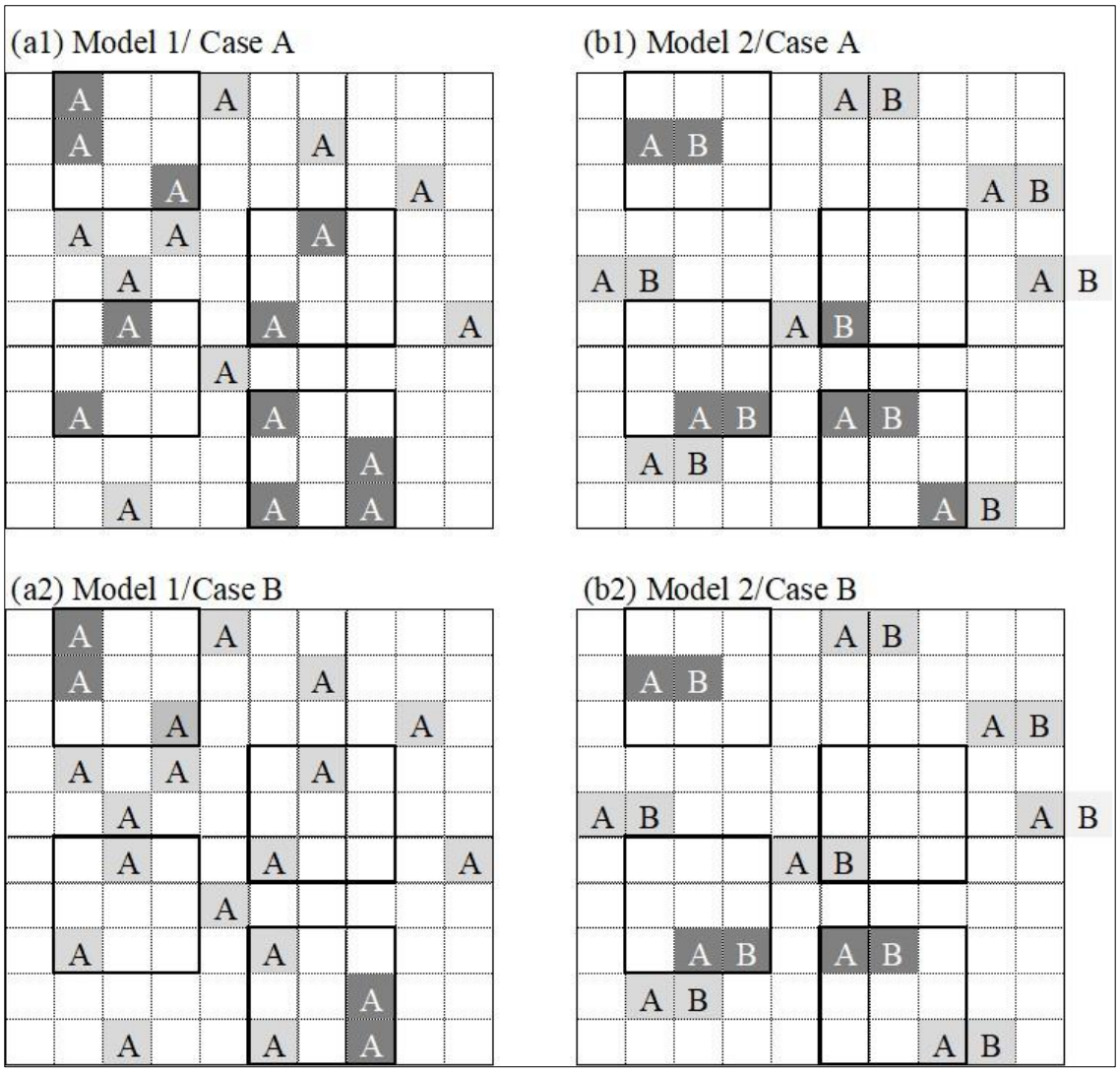

Figure 1 Schematic illustration of models 1 and 2, in which molecules of TLR4 exist (a) individually and (b) in pair, respectively, on the cell surface. Shaded lattices indicate the presence of TLR4, and the darkly shaded ones indicate the activated TLR4. The thick square indicates the region binding with the LPS aggregates.

3. Results

3.1. Monomeric LPS activates TLR4 most effectively in single-molecule-activation model (Case A)

Figure 2 shows the relationship between the size of the LPS aggregates, m, and the number of activated TLR4 at various existence probabilities of TLR4 for case A, at the total number of LPS molecules C = 729. The simulation results for models 1 and 2 were, of course, almost the same. The higher the existence probability of TLR4, the more molecules of TLR4 were activated. At any existence probability of TLR4, the smaller the size of LPS aggregates (the more aggregates), simply the more molecules of TLR4 were activated.

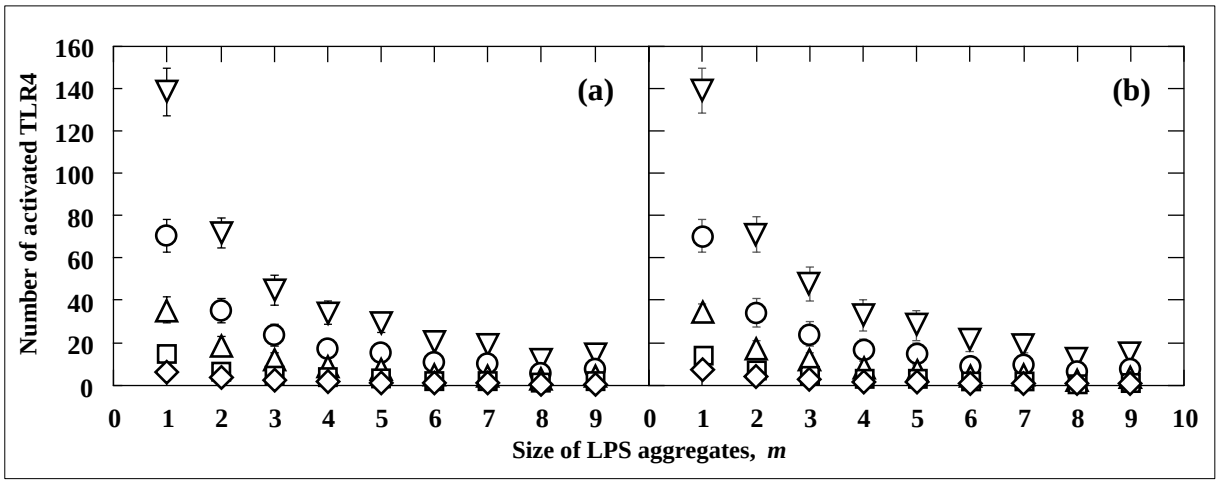

Figure 2 Effect of the size of the LPS aggregates, *m*, on the number of activated TLR4 at various existence probabilities of TLR4 for case A in (a) model 1 and (b) model 2. The existence probabilities of TLR4 are (◇) 0.01, (□) 0.02, (△) 0.05, (○) 0.1 and (▽) 0.2. The total number of LPS molecules *C* is 729. Symbols and bars indicate the mean and standard deviation, respectively, for 50 times attempts.

3.2. Monomeric LAL is not the most effective form to activate TLR4 in dimerized-molecule-activation model (Case B).

Figure 3 also shows the relationship between the size of the LPS aggregates, *m,* and the number of activated TLR4 at various existence probabilities of TLR4 for case B. The total number of LPS molecules was the same (C = 729) as in case A. The higher the existence probability of TLR4, the more the molecules of TLR4 were activated. At any existence probability of TLR4, the smaller the size of LPS aggregates, the more molecules of TLR4 were activated. However, when LPS did not form aggregates and existed as monomers (m = 1), the number of activated TLR4 decreased. The number of activated TLR4 at a certain existence probability of TLR4 was higher in model 2 than in model 1. The effect of LPS aggregation size on TLR4 activation was similar in both the models.

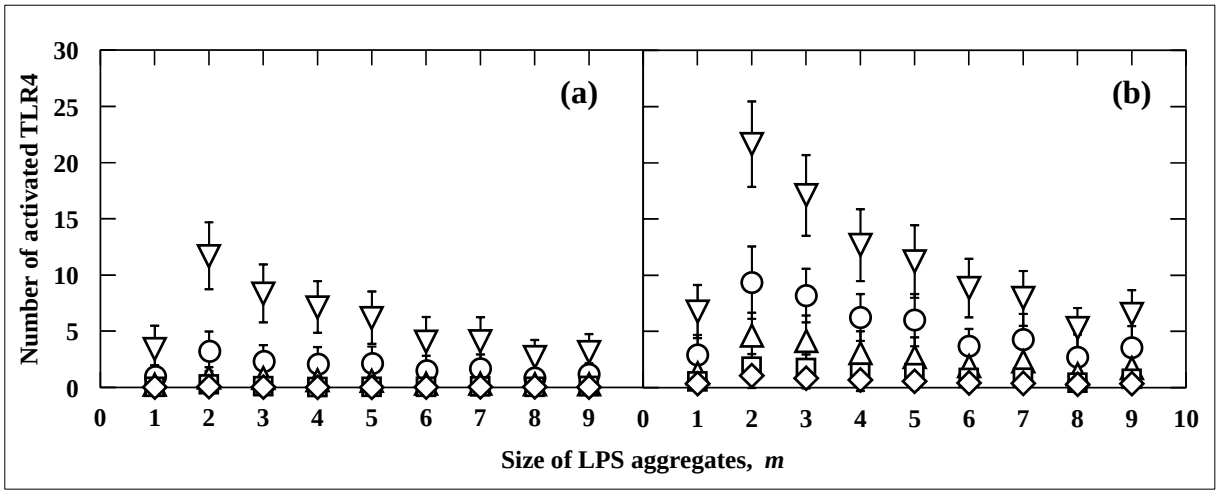

Figure 3 The relationships between the size of LPS aggregates, *m*, and the number of activated TLR4 at various existence probabilities of TLR4 for case B in (a) model 1 and (b) model 2. The total number of LPS molecules *C* is 729. The keys are the same as in Figure 2.

3.3. TLR4 activation is correlated with surface area of LPS aggregates except of LPS monomers.

The number of activated TLR4 may depend on the area covered with LPS aggregates. To confirm this, the number of activated TLR4 was plotted against "m^2×Round (C/m^3)", the number of lattices covered with LPS aggregates, at the existence probability of TLR4 of 0.01 and 0.2 in Figure 4. Since m^2×Round (C/m^3) is proportional to $1/m$, the smaller

the LPS aggregates (the larger the number of aggregates), the higher the fraction of activated TLR4. However, when the LPS molecules did not form aggregates and existed as monomers (m = 1), the fraction of the activated TLR4 was low. Those data points are shown as outliers in Figure 4. There can be certain conditions that monomeric LPS is not effective to activate TLR4. Since the relationship between the size of the LPS aggregates and the number of activated TLR4 showed similar trends in both models 1 and 2, only model 2 will be dealt with in the next section.

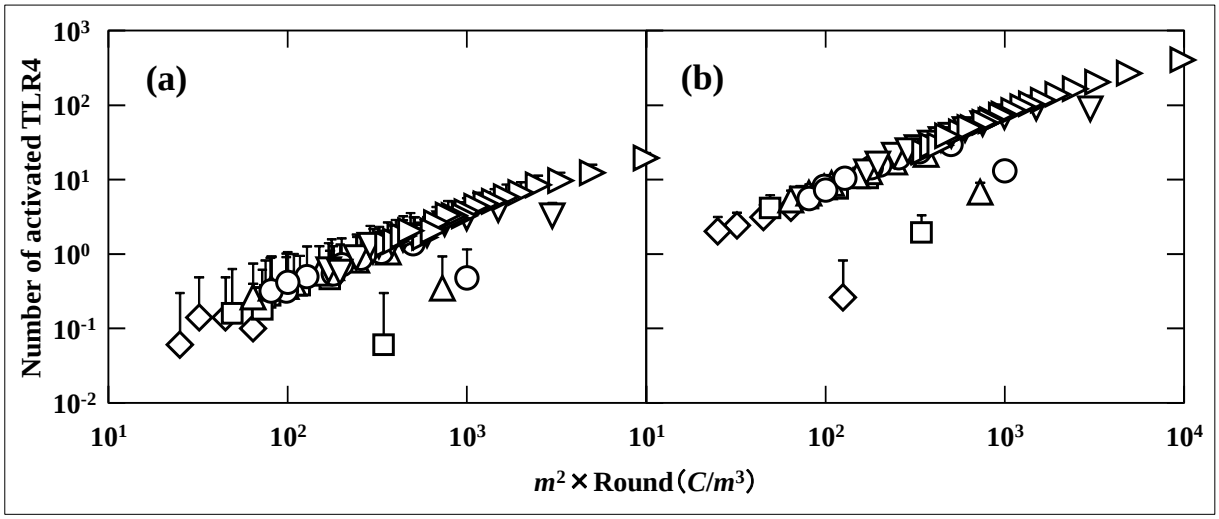

Figure 4 Relationship between the number of activated TLR4 and the number of lattices covered with LPS aggregates, m^2×Round (C/m^3), at the existence probabilities of TLR4 of (a) 0.01 and (b) 0.2 in model 2. The total number of LPS molecules is (◇) 125, (□) 343, (△) 729, (○) 10^3, (▽) 3×10^3, and (▷) 10^4.

4. Discussion

Aurell et al. [17] reported that LPS formed smaller LPS primary lipidic particles at the concentrations several orders of magnitude lower than the critical aggregation concentration, and that there was not any visible sign of the LPS breaking up into individual LPS monomers. This indicates the difficulty of the monomeric LPS preparations.

Takayama et al. reported that monomeric LPS was more active than the aggregated form in the LAL assay and murine peritoneal macrophage response [13], and that the highly disaggregated form of LPS (possibly monomer) was the active unit for the stimulation of the cellular response [14]. Mueller et al. [15] also prepared monomeric LPS, and measured biological activities of the preparations. They did not observe biological activity including the LAL activation. They concluded that aggregates are the biologically active units of LPS. This discrepancy strongly indicates that there is experimental difficulty to prepare and maintain monomeric LPS in solutions. Considering the high hydrophobicity of LPS, which has several fatty acids in one molecule, LPS is probably difficult to keep in monomeric form in water even if it is below the critical aggregation concentration.

Recent study in the LAL activation [7,18,19] strongly suggests that LPS aggregates are essential for the activation of LAL. In other words, monomeric LPS does not activate LAL. Shibata et al. [7] demonstrated that formation of dimers or multimers was necessary for the activation of Factor C on the LPS aggregates. They also indicated that Factor B, another LPS binding protein, was activated on the same LPS aggregates [19]. It is difficult to consider that LPS monomer can provide such a space for the activation of Factor C and Factor B. Miyagawa et al. [20] demonstrated that monomeric LPS could not show the activation pattern of Factor C provided by LPS aggregates by a statistical model. Given monomeric LPS cannot activate Factor C, the LAL test should be negative if the sample contains only monomeric LPS. This finding provided us to re-evaluate the previous studies on monomeric LPS. Although Takayama et al. [14] observed high LAL activity with their preparations of monomeric LPS, their preparations were not monomeric forms because monomeric LPS should not be reactive to the LAL. Assuming that the monomeric LPS was re-aggregated to make small LPS aggregates, it is reasonable that they observed higher activity with their monomeric LPS preparation than aggregated LPS because smaller aggregates were reported to show higher biological activities [21-23]. Mueller et al. [15] did not observe the LAL activation with their monomeric LPS preparations, and the monomeric LPS seemed to be maintained in their preparations.

The difficulty of experiments using monomeric LPS preparations suggests that a simulation using a statistical model is helpful to evaluate the effect of LPS aggregation status on the biological activity. This study is designed to analyze the effect of LPS aggregation status on TLR4 activation by using a statistical model. This analysis can also provide us the possible causes of the similarity between the LAL test and the rabbit pyrogen test.

When LPS is administrated in the human or animal blood stream, fever is usually observed on a dose-dependent basis. Fever is a symptom of inflammation or infection, and extremely high-dose LPS administration can cause multiple organ failure or even death [24]. Inflammation is probably a controlled feverish condition by the immune system, and multiple organ failure is obviously caused by overwhelming the control of the inflammation or cytokine production. Therefore, it is useful to analyze the controlled feverish condition to understand the control system for cytokine production by LPS, and the rabbit pyrogen test condition is just in this situation.

To simulate the TLR4 activation, the model was simplified under some assumptions. One was the binding of LPS aggregates with a target cell when they were encountered. Although the LPS transfer from LPS aggregates to TRL4-MD2 involves at least 2 steps, binding of LBP to LPS aggregates and transfer LPS monomers to CD14, it could be simplified to be one step transfer from LPS aggregates to the cell surface if LPS aggregates are binding to the cell. The amounts of dispersed free CD14/LPS complex from the bound LPS aggregates can be ignored because the LPS aggregates and the cell are close enough to transfer LPS monomers to MD2. Risco and Silva [25] reported the binding of LPS aggregates to macrophages. This observation supports the assumption for the simplified model.

The threshold pyrogenic dose (more than or equal to 0.1˚F rise in 50% of volunteers) of standard endotoxin in humans was reported as 4.1±0.55 EU/kg, and the 8 EU/kg dose peaked at 4 h with a mean temperature rise of 1.9˚F [26]. When LPS is administrated in the human blood stream at 10 EU/kg (1 ng/kg), the concentration of LPS can be calculated as 1.3×10^{-12} mol/L or 0.44 - 1.2×10^{2} molecules/white blood cell, assuming that the blood volume, the numbers of white blood cells in blood, and the LPS molecular weight are 0.077 L/kg body weight, 3.5 - 9.5×10^{9} cells/L, and 10,000, respectively. Because a feverish condition with 10 EU/kg is one of the minimal mild inflammatory states in humans, an LPS dose between 10 EU/kg and 100 EU/kg can be considered as a typical condition for fever or inflammation. Therefore, the suitable condition for this study is estimated as 10^{2} - 10^{3} LPS molecules/white blood cell. Given the size of monocyte is 30 μm, there should be enough surface area for the LPS aggregates that can be 60 - 1200 nm [27]. Therefore, one of our assumptions that LPS molecules are transferred from LPS aggregates to the surface of white blood cells can be used, and this simplifies the model of this study. Moreover, a condition with C between 10^{2} and 10^{3} is suitable to analyze the inflammatory response.

Since recent studies revealed that dimerization of TLR4-MD2-LPS complexes is necessary for activation, the dimerized-molecule-activation model (Case B) is considered as the real model for the TLR4 activation, not the single-molecule-activation model (Case A). The most remarkable difference between Case A and Case B was observed at m = 1. While the highest TLR4 activation was observed at m = 1 in Case A (Figure 2), suppression of TRL4 activation was observed in Case B (Figure 3). Case A also showed higher activation rates of TLR4 than Case B. These results suggest that the real model (Case B) suppresses unexpected TLR4 activation, especially when LPS monomers with soluble CD14 are spread out in the blood stream. Then the TLR4 activation is localized at the area close to LPS aggregates. This is reasonable for the strategy of the mammalian defense system to localize the inflammation caused by infection. These results support that aggregates are the biologically active units, not monomeric LPS in the early stage of inflammation or infection.

Figure 3 (Case B) shows the relationship between the size of the LPS aggregates and the number of activated TLR4 molecules at C = 729, that is between 10^{2} and 10^{3}. Even though smaller aggregates provided higher TLR4 activation than larger ones, TLR4 activation was low with monomeric LPS. This suggests that the innate immune system discriminates monomeric LPS from LPS aggregates. The efficiency of TLR4 activation by LPS aggregates can be correlated to the surface area of the LPS aggregates, and Figure 4 confirmed this showing the increase of TLR4 activation according to increase of total area that assumed to contact with the cell. In Figure 4, there were several data points out of the trend, and they were the data at m = 1. This is further evidence that the innate immune system discriminates monomeric LPS from LPS aggregates. The difference in TLR4 activation between monomeric and aggregated LPS decreased according to the increase of the LPS numbers, and there was not significant difference at $C = 10^{4}$. This suggests that too much LPS causes uncontrollable activation of TLR4. This can be the situation of severe sepsis and multiple organ failure.

The expression of TLR4 is increased by the stimulation of LPS, and the increase of TLR4 expression enhances TLR4 activation in response to stimulation by LPS [28, 29]. In this study, increased expression of TLR4 provided higher TLR4 activation (Figures 3 and 4). The results of this study supported those studies.

Positive correlations have been observed between the LAL test and the rabbit pyrogen test [4]. The rabbit pyrogen test is based on cytokine production through TLR activation [6], and is different in mechanism from the LAL test, which is initiated by the Factor C activation by LPS. One of the most possible reasons for the correlation is the similarity of the reduced reactivity to monomeric LPS. The reduction of the reactivity to LPS monomers is caused by the dimerization of the receptors, Factor C and TLR4-MD2. Therefore, low monomeric LPS activity caused by the dimerization of receptors is one of the most important factors in the similarity between the LAL test (horseshoe carab coagulation system) and the rabbit pyrogen test (animal TRL4 activation system). LAL is based on the coagulation of blood from the horseshoe crab, and fever in rabbits is caused by cytokine production through TLR4 activation. Both reactions are defense systems for the animals, and need to be localized. Unexpected systemic coagulation or inflammation should be avoided from the view of the defense system from infections. Inertness to monomeric LPS can prevent the unexpected spread of the reactions in the whole system.

5. Conclusion

The dimerization of the receptors reduces the activity of monomeric LPS in the horseshoe crab coagulation system and the mammal TLR4 activation. This is the key factor of the similarity between the LAL test and the rabbit pyrogen test. This can also explain the mechanism of the localization of inflammation and coagulation in the animal defense system.

Compliance with ethical standards

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Disclosure of conflict of interest

Dr. Masakazu Tsuchiya is a Senior Research Scientist at Charles River Laboratories, which manufactures LAL. No other competing financial interests exist.

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