

Possible Mechanism of Low Endotoxin Recovery

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Introduction

Low Endotoxin Recovery (LER) was first presented by Dr. Joseph Chen in April 2013.¹ A masking effect of endotoxin was observed with a matrix containing a chelating agent and a detergent, and the endotoxin potency was not successfully recovered by dilution or magnesium replacement, the usual way to overcome the inhibition caused by chelation. The LER brought us some concerns relating to the suitability of the Limulus Amebocyte Lysate (LAL) test for the endotoxin detection:

1. Endotoxin activity may be reversibly altered under certain conditions. Therefore, there is a risk of appearance of hidden endotoxin activity.
2. The LAL test may not correlate with pyrogenicity.
3. The Bacterial Endotoxins Test (BET) may underestimate endotoxin activity in products if there is the LER.

Since the US Food and Drug Administration (FDA) recommended the establishment of procedures for storing and handling samples for bacterial endotoxins analysis using laboratory data in Question #3 of the FDA Guidance,² a hold-time study is considered to be necessary for each product. Even though FDA pointed out the possibility of a difference in reactivity between purified bacterial endotoxin and native sources of endotoxin, a purified endotoxin, such as control standard endotoxin (CSE) and US Reference Standard Endotoxin (RSE), is usually used for the hold-time study. CSE and RSE are prepared from purified lipopolysaccharide (LPS) and additives. The LER is a potency change of purified LPS by a chelating agent and a detergent. The mechanism of the LER is not fully elucidated, but it is anticipated that the removal of divalent cations by a chelating agent and dispersing LPS by a detergent are involved in the LER.

LPS is an amphipathic molecule and exists as aggregates in water.³ Since the degree of LPS aggregation is one of the factors determining the biological activities of endotoxin, many researchers reported the relationship between the morphological structures and the biological activities of LPS. Since the risks of the LER relate to the reversible biological activity change of endotoxin, it is worth reviewing previous papers on the aggregation of LPS and its biological activity. The purpose of this article is to discuss the potency change of endotoxin in the LER by reviewing previous studies.

LPS Aggregation

LPS consists of a hydrophilic polysaccharide portion and a hydrophobic Lipid A portion.⁴ Critical research has shown that the endotoxically active nucleus is the Lipid A portion of LPS.³ The molecular weight of the LPS monomer is about 10 000 to 20 000,⁵ and is not consistent because of the variety of numbers of the repeating unit of the sugar chain in polysaccharide portion. This indicates that endotoxin or LPS is not a consistent single molecule, but represents a group of substances found in the cell wall of Gram-negative bacteria. Moreover, LPS forms aggregates in solutions,^{3,6} and the apparent molecular weights of LPS aggregates are estimated as 300 000 to 1 000 000.⁵ A water suspension of purified *E. coli* LPS did not pass through 0.025 µm and 1000 kDa molecular cut filters.⁷ This result also supported that the active LPS forms aggregates in water. The ultrastructural morphology of LPS was reviewed well by Brogden.⁶ The morphology of LPS is affected by physical (lyophilization, sonication, heating, etc) and chemical (detergents, pH, metal cations, etc) conditions.

LPS aggregation can be dissociated by deoxycholate (DOC) to monomers.^{8,9} They showed the sedimentation coefficients of LPS preparations were reduced to around 1S (Svedberg unit) by addition of DOC (Table 1). The molecular weights of *E. coli* LPS preparations in 2% DOC were estimated as 8820 to 20 700.⁸ This indicated that DOC dispersed LPS to the monomer level. The filtration study by Sweadner et al.⁷ also showed 100% penetration of LPS in 1% DOC through 10K Da and 100K Da molecular cut filters, which did not allow LPS in water passing through. This supported the hypothesis that DOC broke down LPS aggregates to the monomer level. Interestingly, dialysis resulted in a reassembly of the LPS structure to larger sizes (Table 1).^{8,9} Therefore, the degrees of LPS aggregation can be reversibly changed by DOC.

Galanos and Luderitz¹⁰ demonstrated that the degrees of LPS aggregation were altered in different salt forms. The sedimentation coefficient of LPS was smallest in triethylamine salt form, and the aggregation sizes of LPS were very large in magnesium and calcium salts. They used electrodialysis to obtain LPS in uniform salt forms. Conversion from the original LPS to a triethylamine salt form was not achieved by the simple addition of triethylamine.

Table 1. Change in Degree of Aggregation of LPS by Sodium Deoxycholate

LPS Source	Sedimentation Coefficient		
	Water or Tris	DOC	After Dialysis
<i>E. coli</i> O111:B4	73	0.94	17.9
<i>E. coli</i> O113	NM	1.04	12.8
<i>Shigella flexneri</i>	112	1.48	7.5
<i>Salmonella typhimurium</i>	110	1.40	11+20
<i>Salmonella minnesota</i>	60+106	1.48	21.5
<i>S. minnesota</i> Rd1P-	134+186	0.91	34+77

DOC: 2% to 3% Sodium Deoxycholate
 NM: not measurable

LPS Aggregation Sizes and Biological Activity

Pyrogenicity was also changed when there were changes in LPS aggregation sizes.⁸ Pyrogenicity of LPS was completely lost in 0.5% to 2% DOC, but was recovered after DOC was reduced by dilution or dialysis (Table 2). Considering the degrees of aggregation of LPS, these results indicate that a minimum size of LPS is necessary to induce pyrogenicity.

Figure 1 shows a plotting of the Fever Indices (FI) against the sedimentation coefficients of LPS reported in Ribi et al.⁸ A threshold of the aggregation size of LPS was observed to show high pyrogenicity. Considering this relationship, LPS must be aggregated to cause fever.

Komuro, et al. demonstrated that sonication reduced the aggregation sizes of LPS, and that there was an optimum size to express pyrogenicity and the LAL reactivity.¹¹ They observed pyrogenicity in small sizes of LPS aggregations. This result may be discrepant to Ribi et al.⁸ They pointed out the possibility of masking of the LPS active sites by detergents. There is also a possibility of partial reassociation of fractionated LPS in water because the LPS fractions did not contain any additives to stabilize the LPS aggregation condition.

The LPS in triethylamine salt form showed smaller sizes of aggregation and higher pyrogenicity.³ The sedimentation coefficients of LPS in

Table 2. Change in Pyrogenicity of LPS by Sodium Deoxycholate

LPS Source	Fever Index			
	Saline	DOC	Dilution	Dialysis
<i>Salmonella enteritidis</i>	29.6	0.0	34.5	20.7
<i>E. coli</i>	32.5	1.6	49.6	50.8
<i>Bordetella pertussis</i>	21.8	0.0	22.8	28.5

DOC: 0.5% to 2% Sodium Deoxycholate
 Dilution: LPS in DOC was diluted with saline.
 Dialysis: LPS in DOC was dialyzed against saline.
 LPS dose: 0.1 mg for *S. enteritidis*, 0.25 mg for *E. coli*, and 0.5 mg for *B. pertussis*.
 Fever Index: Area under the fever curve.

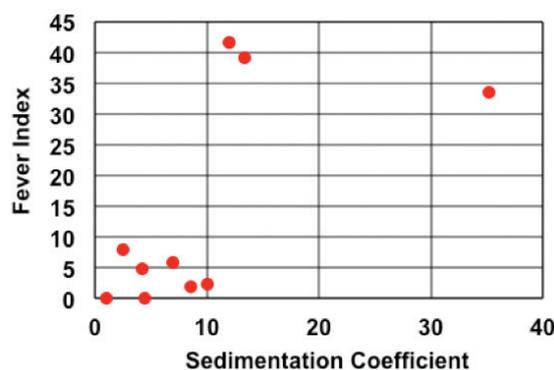


Figure 1. Correlation between Pyrogenicity and Sedimentation Coefficient of LPS.

triethylamine salt form were around the threshold for expression of pyrogenicity in Figure 1. The results suggested that the biological activity of LPS was affected by the solubility of LPS even though the aggregation is necessary to express biological activity.

Mueller et al. suggested that aggregates are the active units of endotoxin.¹² Monomer solutions of Lipid A were separated from aggregates by using a dialysis diaphragm with a cut-off of 5000 Da. The Lipid A used was in triethylamine salt form, and its molecular weight should be <5000, probably around 2000. They compared aggregates and monomers of Lipid A in biological activities, such as TNF- α production from human mononuclear cells and the LAL test, and observed activity only with the aggregates.

Considering these studies, the degree of aggregation of LPS affects its biological activity, monomers of LPS does not show biological activity, aggregates can show biological activity, and there is an optimum size of LPS aggregates to express biological activity.

LPS Aggregation in LER Conditions

Hannecart-Pokorni et al. reported LPS aggregation change in citrate and polysorbate in 1974.⁹ A combination of citrate and polysorbate is one of the LER conditions. The sedimentation coefficients of LPS were around 10S in citrate and polysorbate or sodium deoxysulfate (SDS), even though DOC dispersed the LPS to around 1.5S (Table 3). The sedimentation coefficient of 10S is around the threshold to express pyrogenicity in Figure 1. Interestingly, the sedimentation coefficients of LPS in citrate and polysorbate were not recovered to the larger sizes after dialysis, even though those in DOC were significantly increased. This suggested that the structure of LPS altered by citrate and polysorbate is not easily recovered. This agrees with the observation in LER. Citrate probably removes divalent cations, and polysorbate disperses LPS molecules. Divalent cations, such as magnesium and calcium, are important to maintain the structure of LPS aggregation, and detergents can easily pick up LPS molecules from the LPS aggregates that are weakened by removal of divalent cations.

Table 3. Effects of Detergents on LPS Aggregation

LPS Score	Dialysis	Sedimentation Coefficient			
		Water/Tris	DOC	Cit/Tween	2% SDS
<i>Shigella flexneri</i>	Before	112	1.48	10.2	7.5
	After		7.5	7.6	8+10
<i>Salmonella typhimurium</i>	Before	110	1.40	7.9	7.2
	After		11+20	8+12	10.7
<i>Salmonella minnesota</i>	Before	60+106	1.48	9.4	5.6
	After		21.5	10.8	7.8

DOC: 2% to 3% Sodium Deoxycholate
 Cit/Tween: 0.5% Citrate and 0.5% Polyoxyethylsorbitmonolaurate
 SDS: 2% Sodium dodecylsulfate

Reversibility of Biological Activity

Ribi et al. reported the effect of human plasma on pyrogenicity of LPS in DOC.⁸ They demonstrated that LPS potency in DOC was recovered after dilution. However, the potency was not recovered by dilution if human plasma was added to the LPS in DOC (Table 4). DOC dispersed LPS in monomer levels, and direct injection of LPS in DOC did not show pyrogenicity. These results suggested that the human plasma prevented the reassembly of LPS by dilution.

If the potency of LPS in LER conditions is recovered when it is administered into a human, it can cause a safety problem. As Hannecart-Pokorni, et al. demonstrated that LPS aggregation sizes in citrate and polysorbate were not changed by dialysis,⁹ the biological activity of LPS in LER conditions might not be easily changed. They also reported that chloroform treatment recovered the degrees of LPS aggregation.⁹ However, a treatment with an organic solvent is not relevant to *in vivo* conditions. Considering these results, the potency of LPS in LER conditions is not likely to be recovered *in vivo*, and the LER seems not to be a safety problem.

Correlation between Pyrogenicity and the LAL Test

The correlation between the rabbit pyrogen test and the LAL test is widely observed.¹³⁻¹⁹ The BET was established by this recognition of the correlation between rabbit pyrogen test and the LAL test. One of the major reasons for the adoption of the BET was that fewer false negatives were expected in the LAL test than in the rabbit pyrogen test. Wachtel and Tsuji reported that the LAL test is 3 to 300 times more sensitive than USP Rabbit Pyrogen Test and that both methods were correlated well when the endotoxin was pure and undegraded.²⁰ They also observed discrepancies in the correlation using degraded endotoxin with different LAL preparations. They recommended proper selection of an LAL preparation for its application. Most of the LAL preparations in their study are not currently commercially available. It is probably because the current LAL reagents have been improved and selected for the purpose of the BET.

Table 4. Effect of Human Plasma on Pyrogenicity of LPS in Sodium Deoxycholate

DOC (%)	Plasma (mL)	FI After Dilution
0.0	0.0	29.6
0.0	1.0	36.2
0.5	0.0	35.9
0.5	0.25	1.9
0.5	1.0	0.0

DOC: Sodium Deoxycholate
 Plasma: human plasma
 FI after dilution: Fever Index of LPS in DOC/Plasma diluted with saline.
 LPS dose: 0.1 mg for *S. enteritidis*.

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Since the LAL test and the rabbit pyrogen test are biological assay methods, the variability of the methods should be larger than typical chemical analysis methods. Moreover, an endotoxin can cause variability because of its inconsistency in the degrees of aggregation, components of Lipid A portions, and molecular weights. An understanding of these facts is necessary for the appropriate application of the BET.

Discussion

Previous studies demonstrated that the degrees of LPS aggregation were reversibly altered by chemical and physical conditions. Since the degrees of LPS aggregation reflected biological activity, such as pyrogenicity and the LAL reactivity, the biological activity of LPS could be reversibly changed. However, the LPS aggregation change seems not to occur in human blood because such conditions are not found *in vivo*. Human plasma also helps to preserve the potency of endotoxin. For the safety test for endotoxin, it is the potency to endotoxin in product that should be measured. This means that a possible potency of endotoxin in products should not be considered as the target of the BET, although the amounts of endotoxin in products should be controlled under the process control.

LER is a phenomenon of LPS potency change. Even though the LER was recently reported, similar phenomena have been observed. For example, trace amounts of certain metal cations and antibiotics could decrease the potency of endotoxin.²¹⁻²³ The mechanism of the phenomena is not clear, but it may be a change in LPS aggregation to larger sizes, considering LPS in magnesium and calcium salts were partially insoluble in water.⁴⁻¹⁰ On the other hand, the mechanism of the LER caused by a chelating agent and a detergent is probably a change in LPS aggregation to smaller sizes. Although the mechanisms of the LER and the LER-like phenomena may be different, changes in LPS potency are observed as the result of both cases.

Considering the 50-year-experience with the LAL test and the sensitivity of the LAL test, the LER is not a safety issue in the BET.

Endotoxin in products should be measured as the potency in the product. The possible potency of endotoxin in products should not be a measure of the safety because it is variable in a wide range. Recovery of LPS potency by a treatment does not mean that the degree of LPS aggregation was recovered as same as the original LPS. Pyrogenicity of the LPS in DOC was recovered by dilution or dialysis, but the sedimentation coefficients of LPS were different between before and after the treatment.^{8,9} Most importantly, there is no standard degree of aggregation defined for the standard endotoxin.

The LER is an issue on the hold-time study of the BET samples. One of the major causes of the LER is a matrix containing a chelating agent and a detergent. Practical LER phenomena may also involve protein issues.²⁴ Proteins may cause both interference with the LAL reaction and change in potency of endotoxin. To consider the LER, interference of the LAL reaction should be eliminated. When interference is observed in the BET, we need to consider whether it is interference of the sample with the LAL reaction or the LER. The BET should be performed under the condition without interference of the sample with the LAL reaction. The interference of proteins is usually caused by different mechanisms. Therefore, protein issues should be individually solved. Considering these factors, we need to establish a condition and a suitable endotoxin preparation for the hold-time study.

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Author Biography



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