Aspects of Mechanism in Low Endotoxin Recovery

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Purpose

- To propose possible mechanisms of Low Endotoxin Recovery (LER) by analyzing the characteristics of LER with different preparations of LPS.

Materials

LAL: Endochrome-K (Charles River) for the kinetic chromogenic assay (KCA).
LPS
- Highly purified lipopolysaccharide (LPS) derived from *E. coli* O55 (referred as S-type LPS, LPS Research Institute, Japan)
- Highly purified LPS derived from *Salmonella minnesota* R595 Re (referred as R-type LPS, LPS Research Institute, Japan)
- LPS derived from *E. coli* O55:B5 (List Biological Laboratories, CA)
- RSE: USP Reference Standard Endotoxin

LAL Reagent Water (Charles River)
SWI (lot X): Sterile Water for Irrigation USP (lot G063347)
Sodium citrate, polysorbate 20 (PS20), HEPES (Sigma)

Methods

- **Measurement of activity change in LER**
  This method allows successive measurement even every minute.

  0.1 mL LAL (Endochrome-K) in a tube
  ← 0.1 mL LAL Reagent Water
  ← 0.01 mL sample
  Set on Tube Reader (Toxinometer, Wako, Japan)
  Endotoxin activity was measured with KCA.

- **Regular LAL test**

  0.1 mL LAL (Endochrome-K) in a tube
  ← 0.1 mL Sample
  Set on Tube Reader (Toxinometer, Wako, Japan)
  Endotoxin activity was measured with KCA.

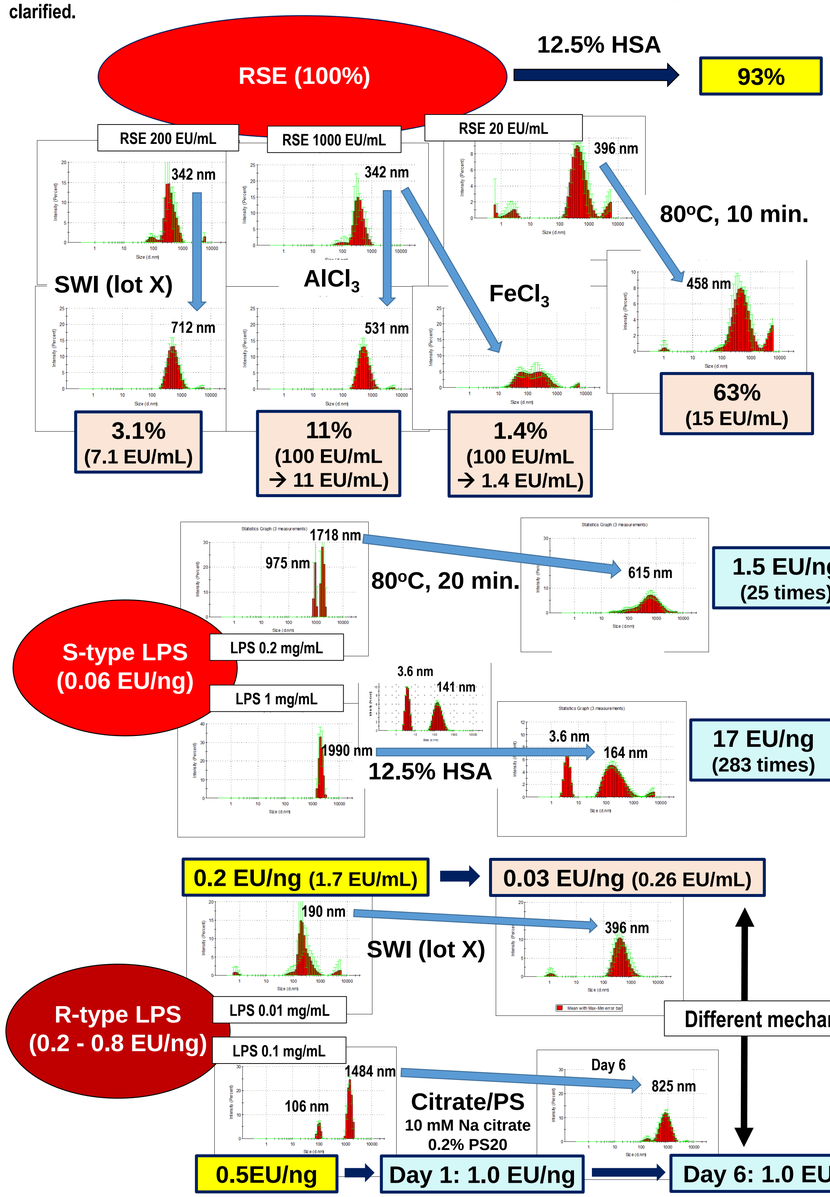
Calculation of LER50
- LER50 in LER was defined as decimal reduction time required at a given condition to reduce 50% of spiked endotoxin activity.
  - Slopes (reaction rate, k) of ln(1/RSE% activity) vs incubation time were calculated.
  - Times required to obtain 50% activity were calculate by the slopes, and used as LER50 values. (LER50 = $ln2/k$ = 0.693/k)
  - Chosen parameters were temperature, pH, salt concentration, citrate concentration, phosphate concentration, and polysorbate concentration.

Dynamic Light Scattering (Zetasizer Nano ZS, Malvern, UK)
- Applicable to substances in liquid
- Detectable wide range of particles in solutions
- No information for actual shapes of substances
- No information for concentrations or amounts of substances

Results and Discussion

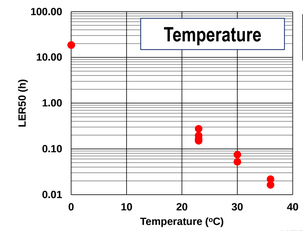
1 Behavior of Different LPS Preparations under LER conditions

Effects of LER conditions on endotoxin activity is dependent on LPS preparations. Initial aggregation states appear to be important for the activity change. Surface conditions of LPS aggregates may affect the activity change. Behavior of endotoxin activity is unpredictable until the LPS source and its aggregation states are clarified.



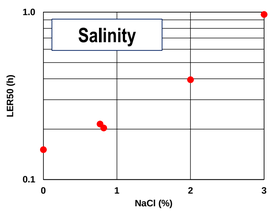
2 Characteristics of Typical LER with RSE

Reaction rates in LER with RSE were affected by temperature, pH, and the concentrations of citrate, polysorbate, and salt. Considering limitation of the ranges for polysorbate and citrate, temperature, pH, salinity should be controlled for hold-time study. To decrease LER effects, lower temperature, higher pH, and higher salinity are recommended.



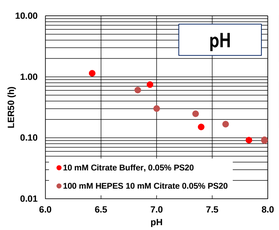
Slope	-0.083
Y-intercept	1.24
r	-0.996

10 mM Na citrate, 0.05% Polysorbate 20, pH 7.3-7.4
Endotoxin in each sample was measured by KCA.



Slope	0.268
Y-intercept	-0.87
r	0.986

10 mM Na citrate, 0.05% Polysorbate 20, 10 EU/mL RSE, pH 7.4
Endotoxin in each sample was measured by KCA.



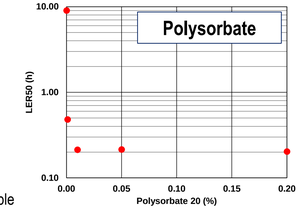
HEPES

Slope	-0.846
Y-intercept	5.57
r	-0.967

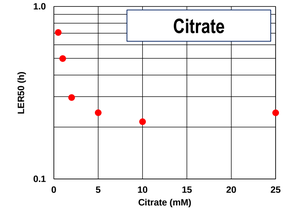
Citrate

Slope	-0.826
Y-intercept	5.36
r	-0.982

10 mM Na citrate, 0.2% Polysorbate 20, 5 EU/mL RSE, Endotoxin in each sample was measured by KCA.



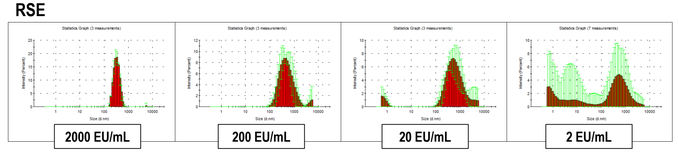
10 mM Na citrate, 10 EU/mL RSE, pH 7.4
Endotoxin in each sample was measured by KCA.



0.05% Polysorbate 20, 5 EU/mL RSE, pH 7.4
Endotoxin in each sample was measured by KCA.

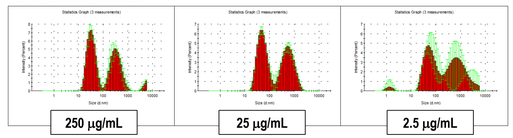
3 Aggregation Change in Dilution

RSE



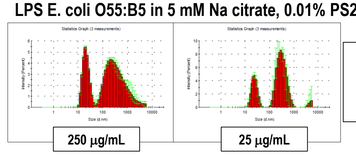
LPS aggregation sizes were changed in accordance with dilution. LPS aggregates seemed to be fluctuated by dilution.

LPS E. coli O55:B5



Endotoxin activity in citrate/PS was less than 10% of the original LPS. The patterns of LPS aggregates displayed limited change, but smaller aggregates seemed to be decreased under the LER condition. The DLS assay was invalid at 2.5 µg/mL for LPS under the LER condition, although the assay was performed with LPS in water at the same concentration. This suggests that the number of LPS aggregates was decreased under the LER condition.

LPS E. coli O55:B5 in 5 mM Na citrate, 0.01% PS20



DLS assay was invalid at 2.5mg/mL.

Conclusion

- **Behavior of endotoxin activity is unpredictable until the LPS source and its aggregation states are clarified.**
- **There may be a different mechanism of endotoxin activity decrease from that of LER caused by citrate/polysorbate.**
- **To decrease LER effects, lower temperature, higher pH, and higher salinity are recommended.**
- **LPS aggregates fluctuated by dilution, and the initial aggregation states are important for LER susceptibility.**
- **The mechanism of LER caused by citrate/polysorbate can be a decrease of active LPS aggregates in the solution.**