ISSN: 2230-9926

Available online at http://www.journalijdr.com

IJDR

International Journal of Development Research
Vol. 10, Issue, 06, pp. 36751-36756, June, 2020
https://doi.org/10.37118/ijdr.19019.05.2020

RESEARCH ARTICLE OPEN ACCESS

INNOVATIVE MECHANISM OF LIMULUS AMEBOCYTE LYSATE ACTIVATION TO ACHIEVE SPECIFICITY AND SENSITIVITY TO ENDOTOXIN; COMPARISON WITH RECOMBINANT FACTOR C REAGENTS

***Masakazu Tsuchiya**

Microbial Solutions, Charles River, Charleston, SC, USA

ARTICLE INFO

Article History:
Received 06th March, 2020
Received in revised form
03rd April, 2020
Accepted 11th May, 2020
Published online 29th June, 2020

Key words:

Endotoxin, Lipopolysaccharide, Limulus amebocyte lysate, Factor C, Factor B, recombinant Factor C, activation mechanism.

****Corresponding author:***
Masakazu Tsuchiya

ABSTRACT

The Limulus Amebocyte Lysate (LAL) test is widely used for detection of endotoxin that is one of the most potent pyrogen. The LAL test is an application of the blood coagulation system that has been considered as a simple enzymatic cascade system. Recent studies revealed that the activation of Factor C, the initial factor for the coagulation is intermolecular autocatalytic reaction on endotoxin aggregates. The second coagulation factor, Factor B also seems not to be activated by simple enzymatic reaction. These activations require endotoxin aggregates. Since Factor B is not activated without endotoxin aggregates, the specificity of the LAL to endotoxin is considered to be achieved by both Factor C and Factor B. This suggested the risk of the use of recombinant Factor C (rFC) reagents that are currently considered to be adopted for endotoxin measurement in the European Pharmacopoeia. There is a difference in amplification of the signals between the LAL and rFC reagents, and the difference may increase false results using rFC reagents.

Copyright © 2020, Masakazu Tsuchiya. *This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.*

Citation: ***Masakazu Tsuchiya.*** "Innovative Mechanism of Limulus Amebocyte Lysate Activation to Achieve Specificity and Sensitivity to Endotoxin; Comparison with Recombinant Factor C Reagents.*", International Journal of Development Research*, 10, 06, 36751-36756

INTRODUCTION

The Bacterial Endotoxins Test (BET) was adopted in 1980 by the United States Pharmacopeia (USP) as the compendial method for detecting contamination in parenteral drugs and medical devices (Chapter <85> USP, 1980). Endotoxins from Gram-negative bacteria, also known as lipopolysaccharides (LPS), are the most common cause of toxic reactions to pharmaceuticals and implanted medical devices contaminated with potential fever-inducing compounds (pyrogens). In over 40 years of testing and usage, the Limulus amebocyte lysate (LAL) test has confirmed the safety of these products, and it is difficult to find a report of failure of detection of endotoxin in those products in the BET's history (Mccullough, 2016).

Research found that the mechanism of the LAL test is a cascade system involving several activations of proteases (Iwanaga, 1992). This cascade system was discovered in the 1980s, and all the factors in the cascade were purified and identified. The LAL test has two pathways; one is triggered by endotoxin and the other is triggered by beta-1,3-glucan (Figure 1). LAL's specificity to endotoxin can be improved by either using beta-glucan blockers (Tsuchiya, 1990 and Bruce, 1999) or removing Factor G, the receptor for the beta-glucan pathway (Obayashi, 1985). The endotoxin pathway involves three serine protease zymogens: Factor C, Factor B, and Proclotting enzyme.

The specificity of LAL to endotoxin has been believed to be achieved by the specific affinity of Factor C to endotoxin, and the following activation of Factors are considered as simple enzymatic reactions. Based on this idea, Tan *et al*., proposed a model for Factor C activation in which a single LPS molecule binds to Factor C to be activated (Tan, 2000). However, recent studies revealed that Factor C is in fact activated by dimerization of Factor C molecules on LPS aggregates (Shibata, 2018). The activation is an intermolecular autocatalytic activation, not a simple enzymatic reaction. The

second factor in the endotoxin pathway is Factor B, and Factor B is also an LPS binding protease zymogen (Kobayashi, 2015). Factor B is activated by activated Factor C on the LPS aggregates. One of the cleavage sites on Factor B is not involved in the protease activity of the activation of Factor C. These findings suggest that the role of Factor B in the cascade system is not only to activate proclotting enzyme, but to increase specificity of LAL to endotoxin.

Recently, recombinant Factor C (rFC) reagents were commercialized for endotoxin measurement (Loverock, 2009 and Bolden, 2017). The specificity of the rFC reagents to endotoxin depends on the specific affinity of Factor C to endotoxin. Because recent studies suggested an important role for Factor B, there may be a difference in specificity to endotoxin between the LAL and the rFC reagents. There is also a difference in amplification of the signals between these reagents. The purpose of this article is to clarify the differences between LAL and rFC reagents according to the recent studies. The rFC reagents are not considered to be as specific as LAL reagents.

Activation of Factor C by Endotoxin/LPS: As stated earlier, Tan *et al*., proposed a model of Factor C activation with binding a of single LPS monomer to a monomer Factor C (Figure 2) (Tan, 2000). This model is different from the one proposed by Shibata *et al*. that requires a dimer or multimer of Factor C on LPS aggregates (Figure 3) (Shibata, 2018). Considering that Tan *et al*., did not show any evidence of existence of monomeric LPS in their experiment, there was a possibility that LPS aggregates reacted with Factor C in their experiments. Nakamura *et al*. showed the dose-response as an optimum LPS concentration in the activation of Factor C (Nakamura, 1988). Miyagawa, *et al*. simulated the activation of Factor C showing the activation pattern is related to the dimerization of Factor C on LPS aggregates (Miyagawa, 2019). This activation pattern was not observed in any LPS aggregates, including monomeric LPS. It can be concluded then that Factor C is not activated by binding of monomeric LPS and a single molecule of Factor C. Shibata *et al*. precisely demonstrated the mechanism of Factor C activation by using several Factor C mutants (Shibata, 2018). Factor C is activated by the cleavage at Phe^{737}in the L-chain. Phenylalanine is one of the cleavage sites for chymotrypsin, which can generate beta-Factor C and shows serine protease activity like alpha-Factor C activated by LPS (Kobayashi, 2014). However, beta-Factor C cannot activate Factor B, the next factor in the LAL cascade system. Interestingly, the activation of Factor C does not need chymotrypsin activity because the cleavage was observed using Factor C mutants in which Phe^{737}residue was substituted with Ala or Glu. Activated Factor C shows trypsin-like protease activity, and Ser^{941} in the L-chain of Factor C is considered the active site. Ser^{941} plays an important role in the LPS-dependent autocatalytic cleavage of the Phe^{737} site because no cleavage at Phe^{737} was observed when the Ser^{941} residue was substituted with Ala. When Phe^{737} residue was substituted with proline, the cleavage was not observed in the mutant, even though it still had active Ser^{941} and LPS binding ability. Because this mutant cleaved at Phe^{737}site in the Factor C mutant in which active Ser^{941} residue was substituted with Ala, Factor C activated intermolecularly. This also suggested that there is an active transition state of zymogen Factor C. For the Factor C activation, LPS is required as a scaffold to induce interaction between the active transition state of Factor C molecules because activation was not observed by using Factor C mutants without LPS' binding ability.

In summary, binding of Factor C to LPS aggregates causes a conformational change resulting in the activation of Factor C forming dimers or multimers that can be intermolecularly activated by cleavage at the Phe^{737} site in the L-chain on LPS aggregates. Therefore, the activation of Factor C is not a simple enzymatic activation, but an intermolecular autocatalytic activation on LAL aggregates.

Activation of Factor B: Factor B is also an LPS binding protease zymogen and is activated by cleavages at $Arg^{103}$and Ile^{124} (Kobayashi, 2015). Arg^{103} can be cleaved by a trypsin like serine protease, but Ile^{124} is unlikely to be cleaved by such proteases. Beta-Factor C activated by chymotrypsin did not activate Factor B (Kobayashi, 2015). This observation suggested that the protease activity of activated Factor C is not enough to activate Factor B. Because Factor B mutant without LPS binding ability was not activated as much as native Factor B, LPS is probably required to activate Factor B by alpha-Factor C (Kobayashi, 2015). These facts suggest that for Factor B to be activated both LPS and alpha-Factor C are required (Figure 3). In other words, Factor B contributes to the specificity of LAL to LPS. Even if Factor C is activated by accident or by proteases, such as chymotrypsin, Factor B prevents further activation of the LAL cascade system without LPS.

LAL and rFC specificity to endotoxin: The specificity of LAL to endotoxin is achieved by the cooperation between Factor C and Factor B on LPS aggregates. Factor C is activated by LPS aggregates, where it reveals a protease activity that cleaves substrates for trypsin-like proteases (Shibata, 2018; Nakamura, 1986; Tokunaga, 1991). The protease activity can be revealed in activated Factor C by chymotrypsin without LPS (Shibata, 2018; Kobayashi, 2014; Tokunaga, 1991). However, this type of activation (beta-Factor C) does not activate Factor B, which requires activated Factor C by LPS aggregates (Shibata, 2018). In other words, Factor B plays an important role in achieving LPS-specific activation of LAL and avoiding the accidental activation of Factor C.

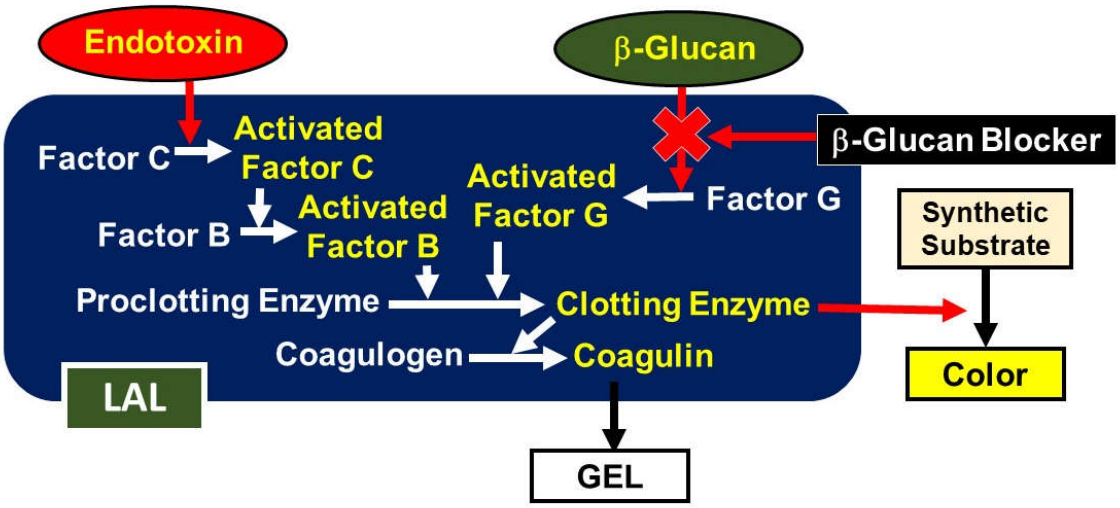
On the other hand, rFC reagents measure the trypsin-like protease activity by using synthetic fluorogenic substrates, such as Boc-Val-Pro-Arg-MCA (Din, 2001). This activity is one of the characteristics of activated Factor C but does not reflect the following Factor B activation. Considering the affinity of Factor C (rFC and natural Factor C) to LPS, its activation is fairly specific, but not as specific as found in LAL.

Amplification of the signal in the LAL test: The dissociation constants of Factor C and Factor B to LPS were reported as 7.58×10^{-10} and 3.49×10^{-9}, respectively (Kobayashi, 2015). The affinity level of Factor C to LPS is similar to that of antigen-antibody binding (Landy, 2015). Considering the highaffinity to LPS, only limited amounts of free activated Factor C and free activated Factor B are available in the reaction mixture. Since activation of Factor B requires both activated Factor C and LPS aggregates, free activated Factor C cannot activate Factor B. Activated Factor B is also maintained on the LPS aggregates, and this suggests that amplification of the signal by circulating activation of Factor B is not expected. Therefore, activation of the Proclotting enzyme plays a major role in amplifying the signal in the LAL test because continuous activation of the Proclotting enzyme can be

Table 1.Summary of Endotoxin Measurement in Natural Water samples using LAL and Recombinant Reagents

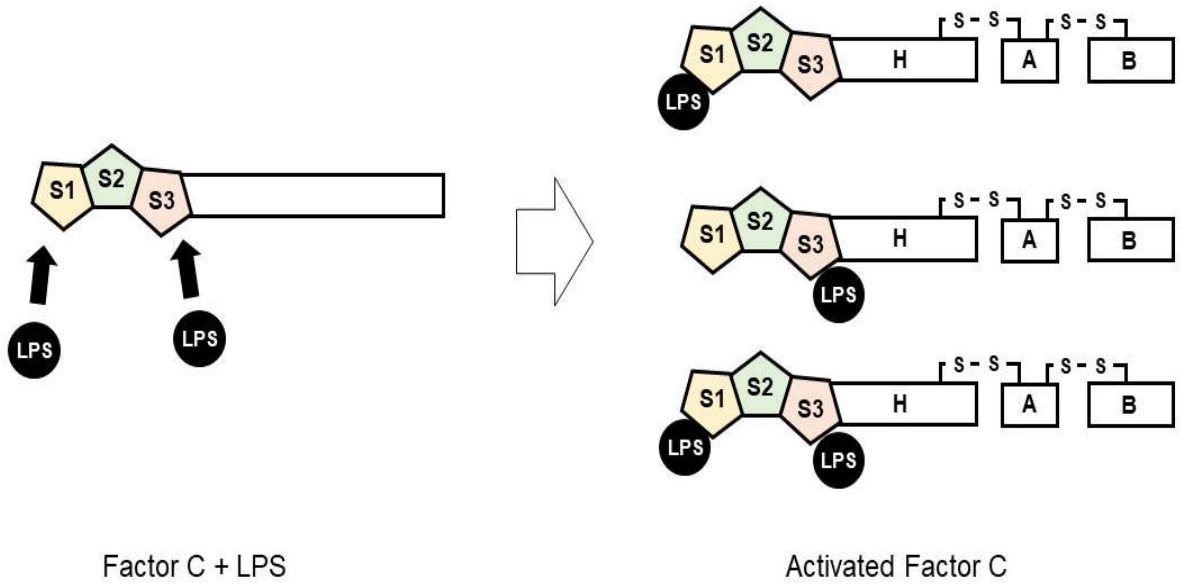
Sample	LAL			rFC			Average rFC
	Endospecy (KCA)	ES-II (KTA)	Kinetic QCL (KCA)	PyroSmart (rTAL)	PyroGene (rFC)	EndoZyme (rFC)	
Lake water	85%	90%	125%	56%	64%	32%	48%
River water 1	87%	97%	116%	96%	91%	53%	72%
River water 2	77%	108%	115%	31%	75%	37%	56%
Domestic waste water	76%	110%	113%	59%	95%	54%	74%
Mineral water	92%	94%	114%	71%	28%	24%	26%
Tap water	72%	97%	131%	91%	43%	11%	27%
Average	82%	99%	119%	67%	66%	35%	51%

*Modified data in Kikuchi, 2017.
*The averages of results using LAL reagents were set as 100%.
*KCA, kinetic chromogenic assay; KTA, kinetic turbidimetric assay; rTAL, recombinant Tachypleus lysate; rFC, recombinant Factor C.



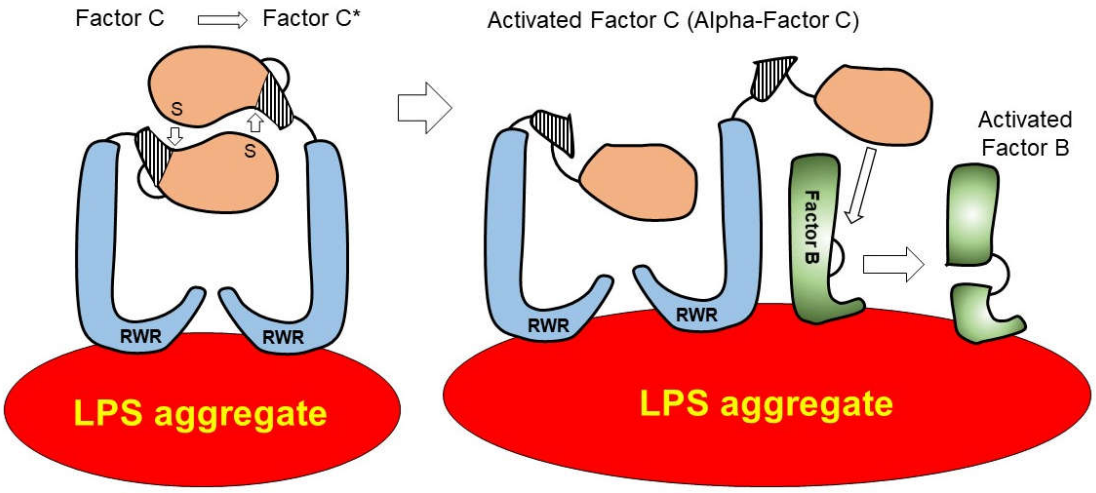
The cascade system in LAL is illustrated, according to the information in Iwanaga, 1992.

Figure 1. Cascade System of Limulus Amebocyte Lysate



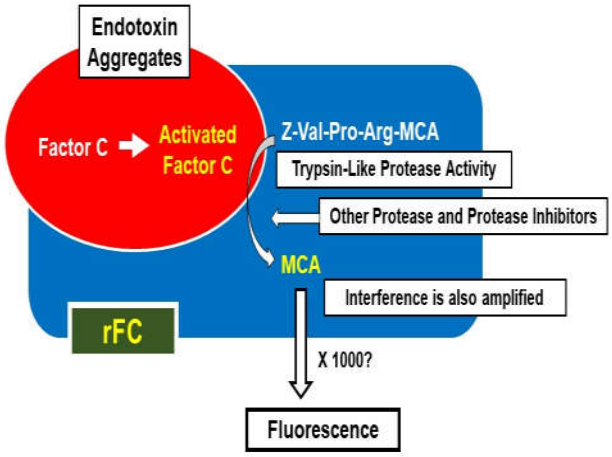
The binding of a single LPS molecule to either Sushi 1 (S1), Sushi 2 (S2), or Sushi 3 (S3) domain would activate Factor C.

Figure 2. Factor C Activation Mechanism Proposed by Tan, 2000



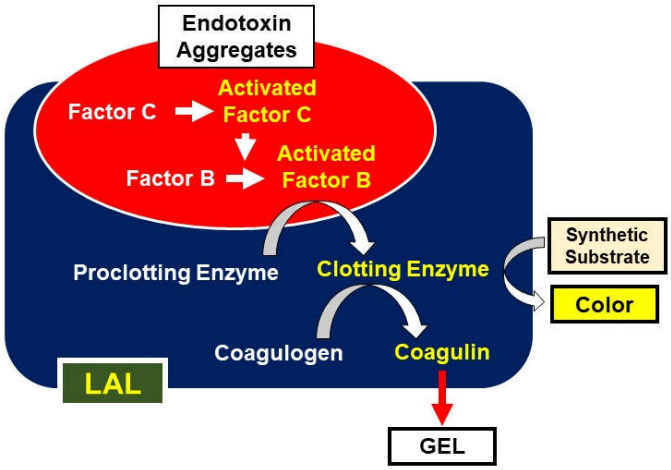
Factor C binds to LPS aggregates, and it causes conformational change in Factor C to Factor C*, the active transition state Factor C. Factor C* forms the dimer or its multimers on LPS, and autocatalytic activation occurs intermolecularly. Factor B binds on the LPS aggregates, and activated by the activated Factor C. These reactions are probably performed on the LPS aggregates, and this increases the specificity of LAL to LPS.

Figure 3. Factor C Activation Mechanism Proposed by Shibata, 2018



Factor C and Factor B are activated on LPS aggregates, and both factors contribute to the specificity of LAL to endotoxin. The signal is amplified after the activation of Proclotting Enzyme

Figure 4. Schematic Diagram of LAL Activation by Endotoxin



Binding affinity of Factor C to endotoxin contributes to the specificity of rFC reagents to endotoxin. The Factor C activity detected by the reagents is a trypsin-like activity cleaving the Arg site of the substrate, and is not the same as the activity for Factor B activation. The substrate can be cleaved by other trypsin-like proteases. The signal is amplified by generation of fluorescence.

Figure 5. Schematic Diagram of Principle of rFC Reagents

expected by activated Factor B. The cascade system of the LAL activation by endotoxin can be illustrated in Figure 4.

Amplification of the signal in recombinant Factor C reagents: Considering the activation of Factor C by LPS aggregates, the amount of activated Factor C is infinitesimal at the LPS concentrations for the BET. The amplification of the signal in rFC reagents is achieved by using fluorogenic substrates. A fluorescent method may amplify a signal by at least 100 times (Kanaoka, 1977), probably 1000 times (Yasothornsrikul, 2000); however, it amplifies other false signals and interference with Factor C protease activity. For example, even a slight amount of a trypsin-type protease can cause false positives, while false negatives can be caused by even a slight amount of coexistent substances that affect the protease activity of the activated Factor C. These false signals are also amplified in rFC reagent assays. Figure 5 shows a schematic diagram of the rFC reagent assay.

Reactivity of Natural-Environmental Endotoxin to LAL and rFC reagents: Kikuchi *et al*. reported more than a 10-fold There were several endotoxin values less than 50% from the average value of the LAL tests, and most of them were results with rFC reagents. The endotoxin in the water samples are considered natural endotoxin, with characteristics that are different from the reference standard prepared from purified LPS from *E. coli* cultured in a nutritious medium. The results suggest that rFC reagents may have less reactivity to a certain type of natural endotoxin. There are several reports to evaluate rFC reagents for the BET (Loverock, 2009; Bolden, 2017; Chen, 2013; Kikuchi, 2018; Muroi, 2019). The rFC reagents were validated by recovery of spiked standard endotox in or purified LPS in samples. These evaluations show conditions met to recover standard endotoxin in the samples but does not guarantee a similar reactivity will occur with natural endotoxin in the samples, as it will when using the LAL test. One of the studies evaluated protein samples containing natural endotoxin, and most of samples (8 of 10) showed endotoxin values within 2-fold range (Chen, 2013). They suggested the effect of beta-glucan in the samples, but there is no relationship between discrepancy of the endotoxin values and

beta-glucan values. There is still a possibility that rFC reagents react differently to a certain natural endotoxin.

DISCUSSION

Recent studies revealed that the activation of LAL is cleverly designed to increase specificity and sensitivity to endotoxin. It is not a simple cascade system composed of simple enzymatic activations. The Factor C activation is an intermolecular autocatalytic reaction through an active transition state responding to LPS (Shibata, 2018). Factor B is activated only when it is on LPS aggregates with the activated Factor C (Kobayashi, 2015). This activation is not caused by trypsin-type protease activity alone, but by the cleavage of Ile^{124}-Ile^{125} in Factor B. This suggests that the reaction is also an inter- or intra-molecular autocatalytic activation. This mechanism prevents the activation of LAL by other trypsin-type proteases. Therefore, the specificity of the LAL test to endotoxin is achieved by the cooperation of Factor C and Factor B. Another important point in the LAL activation is that the amplification of the signal is placed after the activation of Factor B. This increases the reliability of the specificity of LAL to endotoxin.

Use of rFC introduces possible problems in the specificity to endotoxin. These can include false positives caused by contamination of trypsin-type proteases or accidental activation of Factor C, and false negatives caused by inhibitors for the protease activity of activated Factor C. The signal in this assay is the protease activity revealed by the activation of Factor C. However, this type of activity can be found in other trypsin-like proteases. Therefore, the results in rFC reagent assays can be different from those in the LAL test, which has demonstrated its reliability for more than 40 years. Given its importance in the production of parenteral drugs and medical devices, the BET cannot afford false negatives. The study using natural water samples showed that the endotoxin values with rFC reagents were lower than those with LAL (Kikuchi, 2017), suggesting that rFC reagents may provide false negatives. Spike recovery of standard endotoxin is often used for the validation of rFC reagents. However, we should consider the reactivity of natural endotoxin because it is considered a major contaminant in production, while neither standard endotoxin nor purified LPS naturally exists in the environment. One of the supposed advantages of rFC is that there is no reactivity to beta-glucan. In this review, beta-glucan reactivity was not discussed in detail because this subject and the method to avoid it is well-known in the field. Therefore, the reactivity of LAL to beta-glucan is not a current issue in the BET.

In conclusion, the specificity of LAL to endotoxin is achieved by cooperation of Factor C and Factor B, with the latter playing an important role for specificity. The mechanism of rFC reagents is not the same as that of the LAL activation because the protease activity of activated Factor C is not enough to activate Factor B. The endotoxin measurement using rFC can provide a higher possibility of false positives and false negatives, with published data showing the possibility of false negatives in endotoxin measurement using natural water samples. More data with practical samples is necessary to adopt recombinant reagents.

Acknowledgment: The author thank John Dubczak, Alan Hoffmeister, Nicola Reid, Jennifer Boyle, and Rachael Kerr for editing the manuscript.

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

REFERENCES

Bolden J, Smith K 2017. Application of recombinant Factor C reagent for the detection of bacterial endotoxins in pharmaceutical products. *PDA J. Pharm. Sci. Technol*. 71:405-412.

Bruce L 1999. Endotoxin-specific assay, United States Patent 5,998,389.

Chapter <85> Bacterial Endotoxins Test 1980. The United States Phamacopeia 20, The United States Pharmacopeial Convention, Rockville, pp. 888-889.

Chapter <85> Bacterial Endotoxins Test 2018. The United States Phamacopeia 41, The United States Pharmacopeial Convention, Rockville, pp. 6011-6017.

Chen L, Mozier N. 2013. Comparison of Limulus amebocyte lysate test methods for endotoxin measurement in protein solutions. *J. Pharm. Biomed. Anal*. 80:180-185.

Ding JL, Ho B. 2001. A new era in pyrogen testing. *Trends Biotechnol*. 19:277-281.

Iwanaga S, Miyata T, Tokunaga F, Muta T. 1992. Molecular mechanism of hemolymph clotting system in Limulus. *Thromb. Res*. 68:1-32.

Kanaoka Y, Takahashi T, Nakayama H, Takada K, Kimura T, Sakakibara S. 1977. Synthesis of a key fluorogenic amide, L-arginine-4-methylcoumaryl-7-amide (L-Arg-MCA) and its derivatives. Fluorescence assays for trypsin and papain. *Chem. Pharm. Bull*. 25:3126-3128.

Kikuchi Y, Haishima Y, Fukui C, Murai T, Nakagawa Y, Ebisawa A, Matsumura K, Ouchi K, Oda T, Mukai M, Masuda T, Katto Y, Takasuga Y, Takaoka A. 2017. Collaborative study on the bacterial endotoxins test using recombinant Factor C-based procedure for detection of lipopolysaccharides (Part 1). *Pharm. Med. Dev. Reg. Sci*. 48:252-260.

Kikuchi Y, Haishima Y, Fukui C, Nakagawa Y, Ebisawa A, Morioka T, Matsumura K, Ouchi K, Uchida K, Martinez OK, Oda T, Mukai M, Masuda T, Tsukihashi Y, Takasuga Y, Takaoka A. 2018. Collaborative study on the bacterial endotoxins test using recombinant Factor C-based procedure for detection of lipopolysaccharides (Part 2). *Pharm. Med. Dev. Reg. Sci*. 49:708-718.

Kobayashi Y, Shiga T, Shibata T, Sako M, Maenaka K, Koshiba T, Mizumura H, Oda T, Kawabata S 2014. The N-terminal Arg residue is essential for autocatalytic activation of a lipopolysaccharide-responsive protease zymogen. *J. Biol. Chem*. 37:25987–25995.

Kobayashi Y, Takahashi T, Shibata T, Ikeda S, Koshiba T, Mizumura H, Oda T, Kawabata S. 2015. Factor B is the second lipopolysaccharide-binding protease zymogen in the horseshoe crab coagulation cascade. *J. Biol. Chem*. 31:19379-19386.

Landy JP, Ke Y, Yu G, Zhu XD 2015. Measuring affinity constants of 1,450 monoclonal antibodies to peptide targets with a microarray-based label-free assay platform. *J. Immunol Methods* 417:86-96.

Loverock B, Simon B, Burgenson A, Baines A. 2009. A recombinant Factor C procedure for the detection of Gram-negative bacterial endotoxin. *Pharmacopeial Forum* 35:1613-1621.

Mccullough KZ. 2016. Current USP perspectives on low endotoxin recovery (LER). Am. Pharm. Rev. Endotoxin Supl. 2016, 4-7.

Miyagawa Y, Kikuchi K, Tsuchiya M, Adachi S. 2019. A statistical model for activation of Factor C by binding to LPS aggregates. *Eur. Biophys. J.* https://doi.org/10.1007/s00249-019-01400-4.

Muroi M, Ogura N, Mizunuma H, Aketagawa J, Oda T, Tanamoto K (2019). Application of recombinant three-factor chromogenic reagent, PyroSmart, for bacterial endotoxins test field in pharmacopoeia. *Biol. Pharm. Bull*. 42:2024-2037.

Nakamura T, Morita T, Iwanaga S. 1986. Lipopolysaccharide-sensitive serine protease zymogen (factor C) found in Limulus hemocytes. *Eur. J. Biochem*. 154:511-521.

Nakamura T, Tokunaga F, Morita T, Iwanaga S, Kusumoto S, Shiba T, Kobayashi T, Inoue K. 1988. Intracellular serine-protease zymogen, factor C, from horseshoe crab hemocytes, *Eur. J. Biochem*. 176:89–94.

Obayashi T, Tamura H, Tanaka S, Ohki M, Takahashi S, Arai M, Masuda M, Kawai T. 1985. A new chromogenic endotoxin-specific assay using recombined limulus coagulation enzymes and its clinical application, *Clin. Chim. Acta*149:55-65.

Shibata T, Kobayashi Y, Ikeda Y, Kawabata S. 2018. Intermolecular autocatalytic activation of serine protease zymogen factor C through an active transition state responding to lipopolysaccharide. *J. Biol. Chem*. 293:11589-11599.

Tan NS, Ng MLP, Yau YH, Chong PKW, Ho B, Ding JL (2000). Definition of endotoxin binding sites in horseshoe crab Factor C recombinant sushi proteins and neutralization of endotoxin by sushi peptides. *FASEB J*. 14:1801-1813.

Tokunaga F, Nakajima H, Iwanaga S. 1991. Further studies on lipopolysaccharide-sensitive serine protease zymogen (Factor C): Its isolation from limulus Polyphemus hemocytes and identification as an intracellular zymogen activated by a-chymotrypsin, not by trypsin. *J. Biochem*. 109:150-157.

Tsuchiya M, Takaoka A, Tokioka N, Matsuura S 1990. Development of endotoxin-specific Limulus amebocyte lysate test blocking beta-glucan pathway by crboxymethylated curdlan and its application, *Jpn. J. Bacteriol*. 45:903-911.

Yasothornsrikul S, Hook VYH 2000. Detection of proteolytic activity by fluorescent zymogram in-gel assays. *BioTechnol*. 28:1166-1173.

****