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Discussion on the Differences Between Limulus Reagent and Recombinant Factor C Reagent

Masakazu Tsuchiya*

Microbial Solutions, Charles River, Charleston, South Carolina 29407, United States

***Corresponding author:** Masakazu Tsuchiya, Kaz.Tsuchiya@crl.com

Abstract

Limulus amebocyte lysate (LAL) is widely used for the detection of endotoxin, one of the most potent pyrogens. Recent studies revealed the mechanism of activation of Factor C and Factor B, endotoxin-binding proteins in the LAL cascade. It is well-known that Factor C is the first factor to bind endotoxin aggregates. The second coagulation factor, Factor B is important to achieve specificity of LAL to endotoxin because activated Factor C on endotoxin aggregates are essential for its activation. The endotoxin-specific signal is amplified after Factor B activation in LAL. On the other hand, recombinant Factor C reagents only rely on the specificity of Factor C to endotoxin and amplify the trypsin-like activity of activated Factor C that may not be specific to endotoxin. This mechanism seems to be not as specific to endotoxin as LAL. More evaluation and improvement are necessary for recombinant reagents for endotoxin measurement as a safety test.

Keywords

Endotoxin
Limulus amebocyte lysate
Recombinant Factor C
Bacterial endotoxins test

1. Introduction

Limulus reagent, prepared from blood cells of the horseshoe crab, is used for the detection of endotoxin and was listed in various national regulations as an endotoxin test method in the 1980s. This endotoxin assay has been used for the detection of endotoxin in pharmaceuticals and medical devices for 40 years and no incidents of product heat generation due to its failure have been reported [1]. Before the endotoxin test method was adopted, a febrile test was carried out using rabbits, and several comparative studies, including measurements on actual specimens, were carried out to confirm its reliability [2]. As a result, the endotoxin test method was able to detect almost all the specimens that tested positive in the rabbit pyrogenicity test. Endotoxin was also considered to be the most likely and active thermogenic substance to be contaminated in the manufacture of pharmaceuticals and medical devices under GMP regulations, and the endotoxin test method was adopted as an alternative

to the rabbit thermogenicity test. Since then, various improvements have been made to the Limulus test and its performance has been greatly enhanced. However, the specificity and high sensitivity of the Limulus reagent for endotoxin may be the reason it has been trusted as a safety test for drugs and medical devices for such a long time.

The activation of the Limulus reagent is a cascade mechanism in which multiple enzyme precursors are sequentially activated, this was elucidated by a Japanese research group in the 1980s [3]. Recently, details of the activation of the endotoxin-sensing factors, Factor C and Factor B, have been clarified [4,5]. These studies are very interesting in that they show how the specificity of the Limulus reagent for endotoxin is achieved. They suggest that the activation of the Limulus reagent is not a simple enzymatic cascade mechanism as previously thought, but that its specificity for endotoxin is maintained by clever molecular interactions.

Recently, a recombinant Factor C reagent has been developed to measure endotoxin [6]. Recombinant Factor C reagents have also attracted attention in this respect, as there is a move in Europe to ban animal-based testing and the use of animal-derived reagents. This paper compares the specificity and signal amplification mechanisms of the Limulus reagent and the recombinant Factor C reagent.

2. Specificity of the Limulus reagent for endotoxin

The strong and specific affinity of Factor C for endotoxin plays an important role in the specificity of the Limulus reagent for endotoxin. However, the specificity of the Limulus reagent for endotoxin is not ensured by this alone. Recent studies have shown that the activation of Factor C occurs by the molecular interaction of at least two molecules on the endotoxin aggregate, and a single molecule does not cause activation and two molecules can activate each other [4]. Simulation results also support the two-molecule activation model [7]. Studies using various recombinant Factor C mutants have revealed the details of the activation mechanism of Factor C [4]. The activation of Factor C is mediated by the cleavage of Phe^{737}-Ile^{738}. Chymotrypsin is a well-known protease that cleaves phenylalanine, but chymotrypsin can also express the protease activity of Factor C [4-8]. The protease activity of Factor C is trypsin-type activity, usually Val-Pro-Arg-chromophore-like synthetic substrates. Factor C activated by chymotrypsin (β-Factor C) loses its ability to bind endotoxin but expresses the ability to degrade this synthetic substrate. However, this β-Factor C is unable to activate the next factor, Factor B [4,5]. Factor B is only activated when endotoxin-activated Factor C (α-Factor C) and endotoxin coexist. Factor B also has a strong endotoxin-binding capacity and its activation is caused by the same endotoxin as the activated Factor C [5]. In addition, Factor B activation requires two cleavages [5]. Trypsin-type proteases cleave Arg^{103}-Ser^{104} at the first site, but not Ile^{124}-Ile^{125} at the second site. This means that activation is also expected to occur by some kind of intermolecular interaction, indicating that the activation of the Limulus reagent is not a simple enzymatic reaction. This activation of Factor B is probably the most important mechanism by which the Limulus reagent achieves specificity for endotoxin. To avoid false positives due to a simple enzymatic reaction, the activation of Factor B is designed to occur in the presence of activated Factor C and endotoxin. These considerations suggest that the specificity of the Limulus reagent for endotoxin is not only due to the strong affinity of Factor C for endotoxin but also due to the presence of a factor, Factor B.

3. Signal amplification mechanism of the Limulus reagent

The mechanism of highly sensitive measurement of endotoxin by the Limulus reagent has been considered to be signal amplification each time each factor in the cascade mechanism is activated. However, if Factor C

and Factor B are strongly bound to endotoxin, signal amplification by their activation cannot be expected. This means that the signal amplification of the Limulus reagent occurs after the activation of the proclotting enzyme. In the author's experiments, the addition of Factor B to Factor C did not increase the sensitivity to endotoxin, but the addition of the proclotting enzyme increased the sensitivity by approximately 1,000-fold [9]. This means that the signal amplification of the Limulus reagent takes place after the stage in which the proclotting enzyme is converted to a clotting enzyme. This indicates that the signal amplification of the Limulus reagent is performed on endotoxin-specific signals, suggesting the effectiveness of the Limulus reagent in the highly sensitive measurement of endotoxin. The cascade mechanism of the Limulus reagent is shown in **Figure 1** [10].

4. Specificity of recombinant reagents

Recombinant Factor C reagents utilize only Factor C, the endotoxin-sensitive factor of the Limulus reagent, and are based on the principle of measuring the serine protease activity of Factor C activated by endotoxin [6]. Some people argue that the recombinant Factor C reagent has the same reaction as the Limulus reagent because it uses the endotoxin-sensitive factor of the Limulus reagent, but the serine protease activity of the Val-Pro-Arg-chromophore, which is one property of activated Factor C, is hardly the same as the activation of Factor B in the activation of the Limulus reagent. As mentioned above, the activation of Factor B does not occur with β-Factor C, which hydrolyses the Val-Pro-Arg substrate. Factor B is thought to contribute significantly to the specificity of the Limulus reagent for endotoxin. The specificity of the endotoxin assay using only Factor C is lower than that of reagents containing Factor B and proclotting enzymes. Therefore, if recombinant reagents are used to measure endotoxin, a full recombinant reagent containing these three factors should be used.

5. Signal amplification of recombinant Factor C reagents

Currently, commercially available recombinant Factor C reagents provide standard endotoxin detection sensitivity comparable to that of the Limulus reagents. While activation of the proclotting enzyme is the main signal amplification mechanism in the Limulus reagent, the recombinant Factor C reagent uses a fluorescent substrate for signal amplification, as shown in **Figure 2**. Amplifying the slight activity of activated Factor C with a fluorescent substrate also amplifies the influence

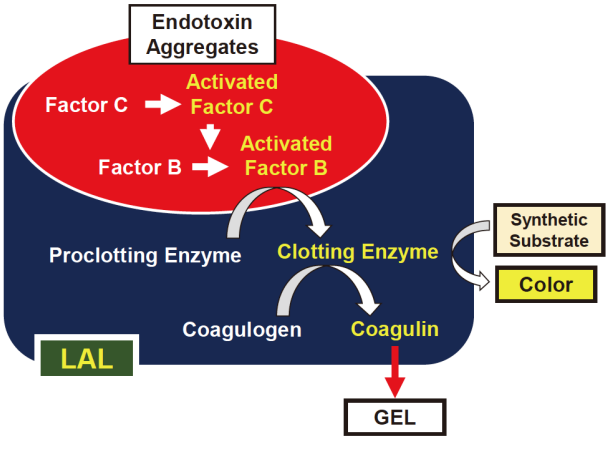

Figure 1. Cascade mechanism of the Limulus reagent [10]. Activation of Factor C and Factor B occurs on endotoxin aggregates and signal amplification takes place after activation of the proclotting enzyme.

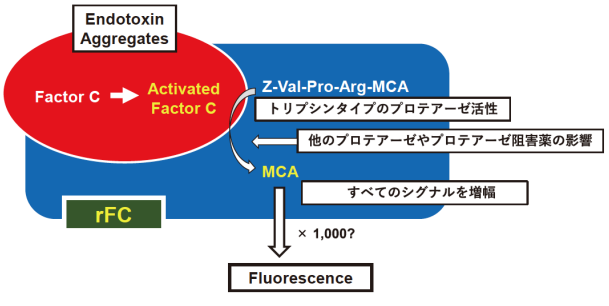

Figure 2. Reaction mechanism of recombinant Factor C reagent [10]. All Factor C-like activities are amplified by fluorescence.

of coexisting substances affecting the measurement. For example, false positives due to the presence of trace amounts of trypsin-type proteases or false negatives due to the presence of protease inhibitors or substances that affect the activity. With the Limulus reagent, such effects are likely to occur after signal amplification, so the effect is likely to be less with the recombinant Factor C reagent.

6. Reactivity of the Limulus and recombinant reagents to natural endotoxins

Recombinant reagents are expected to be an alternative endotoxin test method and attempts have been made to validate them [11,12]. Standard endotoxins are used in these validations, but considering that the endotoxins present in the products are different from standard endotoxins purified from *E. coli*, comparative data using natural endotoxins would be useful. An example of a comparison of a Limulus reagent and a recombinant reagent using natural endotoxin is reported by Kikuchi *et al.* [13]. In this report, the results for natural water showed that many of the measured values of the recombinant reagent were significantly lower than those of the Limulus reagent in lake water, river water, mineral water, and tap water [10]. Our endotoxin measurements in production water also showed a trend towards lower values for the recombinant Factor C reagent [9]. These results indicate that the reactivity ratio of standard endotoxin to natural endotoxin may be different for the Limulus and recombinant reagents, they also suggest the possibility of false negatives due to the recombinant reagent. Further studies need to be conducted to determine which activity is closer to thermogenicity, but if the recombinant Factor C reagent clearly shows lower endotoxin values than the proven endotoxin values of the Limulus reagent, then further investigation may be warranted.

7. Conclusion

In 2018, the European Pharmacopoeia prepared a draft Chapter (EP 2.6.32.) for recombinant Factor C reagents using fluorescence methods, which is expected to be officially listed in 2021. The Japanese Pharmacopoeia and the US Pharmacopoeia have also prepared draft reference information on recombinant reagents (2019), but the recombinant reagents are seen as an alternative method for endotoxin tests. Thus, although different countries have different views on recombinant reagents, it is clear that the use of recombinant reagents will be increasingly considered in the future. However, it must not be forgotten that patient safety is the top priority in endotoxin assays for the safety testing of drugs and medical devices. As presented in this paper, the activation of Limulus reagents by endotoxin is much more complex than previously thought, and recombinant Factor C reagents may produce results different from those of Limulus tests. In order to be able to use recombinant reagents on par with the Limulus test, it is necessary to ensure that they have the same specificity as the Limulus test for a variety of endotoxins. Given the possibility of false negative results with recombinant reagents, as observed in the determination of endotoxin in natural and manufactured waters, recombinant reagents are not a well-established alternative to the Limulus test. To use recombinant reagents in endotoxin testing, further data should be collected on their reactivity to natural endotoxins that may be contaminated, and their safety should be verified. It is hoped that better recombinant reagents will be developed based on future validation.

Disclosure statement

The author declares no conflict of interest.

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