Historical Milestones and Industry Drivers in the Development of Recombinant Lysate for Bacterial Endotoxin Testing

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Introduction

Parenteral pharmaceutical products, ingredient water, and some constituent materials undergo bacterial endotoxin testing since endotoxins are pyrogenic in humans and above a threshold this can induce severe physiological reactions. The conventional method to assess for endotoxin is with the *Limulus* amebocyte lysate (LAL) assay. 2021 sees an easier route to replacing animal derived reagents with non-animal ones, in the form of recombinant lysate and reagents. The use of recombinant lysate refers to the application of a synthetic substitute for the Bacterial Endotoxin Test (BET) using LAL/TAL methodology (the established test uses amebocytes sourced from the *Limulus* or *Tachypleus* horseshoe crabs).[1] This is based on the same *Limulus* clotting factor C protein, produced by genetically modified organisms.[2] However, the 'natural' product and the recombinant variants are not the same in relation to their components or formulation, and this has led to a divergence between compendia.

The idea for the use of recombinant lysate is to provide a reagent that reacts in the same way as the natural cascade within the four species of horseshoe crab: (*Limulus polyphemus*, *Tachypleus tridentatus*, *Tachypleus gigas*, and *Carcinoscorpius rotundicauda*). The cloning technology serves an alternative to the fishing and bleeding of horseshoe crabs (where bleeding is performed through a large dorsal blood sinus called the pericardium). In addition, the use of recombinant technology to substitute the specific pathway seeks to address the concern with some lysates that also detect beta glucans through the activation of factor G. A further advantage with recombinant lysates stems from the production of the test reagent through the use of cell culture. This may result in the supply of a more consistent product.[3]

From around 2003 recombinant lysate has been commercially available, albeit not recognized by any compendia until 2016. However, it has only been in the past few years that manufacturing has become more consistent and subsequently a number of quality control testing laboratories have been looking into the use of recombinant lysate. This somewhat gradual process is attributable to a clash between drivers (seeking to conserve horseshoe crabs, albeit with the mortality rate an imprecise and often contested figure)[4] and points of hesitancy (regulatory uncertainty, lack of standardization, imprecise validation requirements and so on). As of 2021, recombinant lysate will become an established alternative to the traditional LAL test within Europe but within the U.S., when a new chapter appears, a greater level of comparative validation will be required in order to show equivalence. While this disparity is understandable, and this article attempts to show this through a discussion of the historical perspective, the current situation is a disservice to laboratory users. Much of

what has been written on rFC to date has looked at things from a U.S.-centric perspective (rather than U.S. and European) and from a supplier or regulatory perspective; this article embraces the laboratory user perspective.

The LAL Assay

The *Limulus*/*Tachypleus* amebocyte lysate (LAL or TAL) method for the detection of bacterial endotoxin was developed in the 1970s, drawing upon research which began during the mid-1950s with the inquiries of Bang and Levin into Schwartzman-type reactions in horseshoe crabs, triggered as the result of infections with marine-origin *Vibrio* bacteria. The assay was introduced as an alternative method to assess pyrogens, specifically endotoxin derived from largely non-enteric Gram-negative bacteria. This was on the basis that such organisms present the primary pyrogenic risk within pharmaceuticals and healthcare. The LAL assay has largely replaced the rabbit pyrogen test (developed in 1912[5] and entering the USP in 1942, based on Sibert's 1925 test modification,[6] and more recently the test itself has seen a challenge from the Monocyte Activation Test, developed by Poole and colleagues in 1988. With MAT, the amount of cytokine released is a measure of the pyrogenicity of a substance.[7] The merits or otherwise of MAT are outside the scope of this article.

The conventional LAL assay, which first appeared in the 20th edition of the United States Pharmacopeia in 1980 (and was later adopted by the European and Japanese compendia), is based upon a cascade of serine proteases, triggered by trace levels of endotoxin. The culmination of this cascade is a gel clot at the end of the reaction. The primer for the cascade reaction is Factor C (existing as a zymogen).[8] A zymogen is an inactive precursor enzyme zymogen that requires a biochemical change, like a hydrolysis reaction, to change the configuration in order to reveal the active site so that it becomes an active enzyme. Within the horseshoe crab, as Bang first described in 1956,[9] factor C functions as the bio-primer for triggering the coagulation cascade, functioning to alert the arthropod of the presence of a Gram-negative pathogen. In simple terms, as *Limulus* blood cells detect tiny fragments of endotoxin, the animal responds by releasing granules (contained within the type of blood cells called amebocytes) into the surrounding medium. These granules contain a clotting factor, called coagulogen[10] which leads to coagulation. By clotting the immediate surroundings very quickly, the invading bacteria become enmeshed and are therefore stopped from infecting the circulatory system of the horseshoe crab any further.

Initially adopting the LAL assay required validation to be performed in order to replace the rabbit test for pyrogens.[11] The need to demonstrate equivalency faded as the robustness of the LAL test to detect bacterial endotoxin increased. Over time, the LAL method became a test that could be adopted through the route of method suitability testing, and the test also evolved to include turbidimetric and colorimetric variants of the gelation reaction.

Recombinant Technology

In the mid-1990s research began to appear about the possibility of 'laboratory-made' recombinant lysate and reagents, as a replacement for lysate derived from the horseshow crab. There are two interrelated strands of recombinant technology harnessed for the LAL assay: recombinant lysate and a recombinant cascade. The drivers for recombinant technology include variations in the sensitivity and specificity of LAL to endotoxin, and either connected to the variables of supply with horseshoe crabs (conservation linked)[12] or with the European Union focus on moving away from animal derived products and testing (what has been dubbed the 3Rs: replacement, reduction, and refinement of animal testing).[13]

Recombinant lysate

With recombinant lysate, a recombinant protein is an artificially produced (and often purified) protein. The recombiant process occurs, in general terms, when recombinant DNA encoding a protein is introduced into a host organism to express foreign proteins. This process requires the use of specialized expression vectors together with restructuring by foreign coding sequences.[14]

The development of recombinant lysate is based on factor C functioning as the biosensor in the horseshoe crab, reacting to a pathogenic bacterium; and hence the primacy of factor C in the clotting cascade. This made factor C the target with an alternative test based on recombinant technology.[15] Subsequent genetic engineering led to the production of a recombinant factor C protease (rFC). The rFC is a proenzyme inducible by the presence of trace levels of endotoxin.[16] Instead of replicating the entire clotting pathway, the rFC lysate enables activated factor C to cleave a fluorogenic molecule. In turn, this releases a fluorescent signal which leads to the generation of light when endotoxin activates the factor. The signal is proportional to the level of activated factor C and detected through photometry.[17]

An advantage with recombinant lysate is that it does not contain factor G which reacts to (1,3)-β-D-glucan. Hence the lysate will not cause false-positives, in terms of reacting with glucan impurities in finished products.[18] It remains a separate case as to whether detection of glucan is significant or not.

Recombinant cascade

Recombinant cascade reagents (rCR) are different to recombinant lysate in terms of their complexity. Reagents consist of three cloned zymogen proteases (factors C and B and a proclotting enzyme), as part of the formulations. The reagent functions in the assay in a similar way to kinetic chromogenic lysate reagents, where the activated clotting protein cleaves a colorless substrate and this leads to an increase in the intensity of a yellow color.[19]

While the focus has been with BET, the use of recombinant lysate may have other uses, aside from a substitute reagent for the conventional assay. For example, Inoue and colleagues developed a zymogen-based electrochemical sensor. The researchers demonstrated the detection of endotoxin by using recombinant Factor C (rFC). It was found that the activated rFC can hydrolyze a synthetic substrate of Boc-Val-Pro-Arg-p-nitroanilnide to generate an electrochemical active compound, p-nitroaniline (pNA). Released pNA can be

detected by differential pulse voltammetry (at –0.75 V), and this can be translated into equivalent endotoxin units. The idea of zymogen-based electrochemical sensors could provide the basis for new biosensors, as might be used for medical technology or for the assessment of fluid paths.[19]

BET: Development Timeline

Recombinant based alternatives for use with BET have been in development for 25 years. One of the first reported recombinant lysates was an rFC reagent produced from the cDNA of the Mangrove horseshoe crab (*Cacinoscorpius rotundicauda*) in 1995.[20] This pioneering work showed a homology of 97.7% between recombinant factor C and natural factor C; although there were important differences between the restriction sites and subtle base substitutions. A further milestone development with a synthetic substitute to horseshoe crab blood was in 2001, in the form of an improved laboratory-synthesized genetically engineered rFC.[17] In 2003, the first proto-recombinant lysates emerged, at a consistency that could be produced on a commercial scale.[21] However, it remained that wider laboratory tests involving such a replacement were limited due to the availability (production to scale), uncertainty around regulatory opinion, and the market dominance of the current LAL test. Other factors may also have accounted for the slow progression of the recombinant product, such as an unwillingness by laboratories to adopt a single-source of supply; the lack of any standardization (in the form of a pharmacopeia monograph); and uncertainty over the type and extent of validation required to adopt the alternative.

In 2012 progress was made on the regulatory front, with the U.S. Food and Drug Administration (FDA) acknowledging rFC as a potential alternative.[22] With standardization, to provide a protocol for conducting the test, in 2016 a recombinant LAL method was proposed as an addition to the European Pharmacopoeia (in the form of monograph 2.6.32: 'Test for bacterial endotoxins using recombinant factor C'), describing an end-point method. In the same year, the guidance chapter for conducting BET in the European Pharmacopeia was revised to include rFC assays (Chapter 5.1.10 'Guidelines for using the test for bacterial endotoxins') as alternative to classical LAL assays. Drivers for the European chapter development were:

- rFC assay kits from several suppliers are now available.
- Scientific reports detail a wider range of products on which validation has been performed.
- Independent laboratory data has been published with Europe, Japan and the U.S.
- Independent studies have looked at rFC/LAL comparability data with relatively favorable results.
- The first medicinal product released using an rFC assay was approved by the U.S. FDA in 2018.

Following public consultation (January to May 2019), the finalized chapter 2.6.32 appeared in the 3rd supplement to the 10th edition of the Ph. Eur. During July 2020, with an effective date of 1st January 2021 (supplement 10.3 also contains a revised chapter 5.1.10, updated to reflect the new status of rFC-based methods). The structure of the chapter mirrors, in terms of content and layout, the current BET chapter (2.6.14).

The outcome of this development arc is that the European Pharmacopeia has two BET chapters, plus a chapter on MAT and one for the rabbit pyrogen test. While the long-standing BET chapter using LAL (2.6.14) is referenced in individual monographs, chapter 2.6.32 is not. Nonetheless, as chapter 5.1.10 indicates, the replacement of the LAL BET method by rFC is regarded as an approved use of an alternative method.

It was expected by some parties that the United States Pharmacopeia (USP) would follow suit. However, on June 1st, 2020, the USP took a different path and elected not to proceed with an inclusion of recombinant technology in chapter <85> Bacterial Endotoxins. This was because of concerns relating to comparability between lysate sourced from *Limulus* and the recombinant products. The USP position was not that recombinant products are unsuitable, but rather that there was insufficient test data for equivalency and that validation of rFC as an alternative method was required. What is now proposed is for a new chapter 1085.1 to be written. This will require a laboratory seeking to implement rFC to perform comparative validation rather than simply method suitability testing. In Japan, work has begun on studying assay equivalence.[23]

The USP position, which is notably different from the European position, is not shared by manufacturers of recombinant reagents who put forward arguments of equivalence and reliability.[24] In relation to equivalency of rFC with the traditional LAL test, research designed to demonstrate relative efficacy of rFC continues to be generated, such as by Maloney and colleagues[25] and Marius and associates;[26] together with assessments on the detection of different environmental endotoxins.[27] Equally there are other studies that raise concerns over recombinant-based test specificity, including the potential role for Factor B in the clotting reaction.[28] This article acknowledges a difference of opinion and does not set out to adjudicate; it is sufficient to note that manufacturers of recombinant lysate and manufacturers of *Limulus* sourced lysate have differing views on efficacy, equivalency and incontrovertibility of comparative data, as do those who develop compendial texts.

Going forward, however, it is important that the harmonization process between the three major compendia – U.S., European, and Japanese pharmacopeia – so successful in other areas of microbiological testing, is continued in relation to endotoxin testing. Ultimately, it is not in the interests of laboratories to have two separate LAL test monographs, as with the European pharmacopeia (especially where there is no direct statement about equivalency), and the acceptance of recombinant products not being reflected other compendia. This difference presents additional challenges for manufacturers who import into multiple territories. The longer-term goal must be to have a recombinant based assay, ideally one that is kinetic rather than end-point, that is acceptable to all parties and for this to be incorporated into the substantive BET test monographs.

Summary

The acceptance of recombinant lysate for BET takes a step forwards in 2021 with the coming into effect of a European Pharmacopeia chapter describing a BET that uses a rFC based on the gene sequence of the horseshoe crab for the quantification of endotoxins. The test methodology is a fluorometric end-point detection method. This creates a difference with the USP approach, both in terms of the standardization of two methods and the steps required to adopt an rFC test in relation to equivalency and the supporting validation required (where in Europe the method does not have to re-validated, other than in consideration of their use for a specific substance or product; that is. product-specific validation only).

The current state of play means, including a somewhat polarized debate, that clarity for laboratory users is required. The ideal scenario is with a unified BET chapter, containing both methods, and for a harmonized text across the three major compendia. The path to achieving this can be achieved through the wider availability and sharing of data assessing the consistency and robustness of rFC based endotoxin detection assays.

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