Research News

A new era in pyrogen testing

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Pyrogens are substances (usually of biological origin) that cause fever after injection. The best-studied pyrogen is lipopolysaccharide (LPS, also known as endotoxin), found in the membrane of Gram-negative bacteria. During Gram-negative sepsis, endotoxin stimulates host macrophages to release inflammatory cytokines and excessive inflammation causes multiple organ failure and death. Endotoxins are thus ubiquitous pathogenic molecules that are a bane to the pharmaceutical industry and medical community. Limulus amoebocyte lysate (LAL) has been widely used for ~25 years for the detection of endotoxin in quality control of injectable drugs and medical devices. However, variations in sensitivity and specificity of LAL to endotoxin, and the limited supply of limulus (horseshoe crabs) has called for an alternative pyrogen test. Recombinant Factor C (rFC), the endotoxin-inducible coagulation enzyme in LAL, forms the basis of a novel micro-enzymatic assay for high-throughput screens of endotoxin and opens a new era in endotoxin testing. Endotoxin activates the rFC zymogen, which catalytically hydrolyses synthetic substrates to form measurable products, thus quantifying the endotoxin.

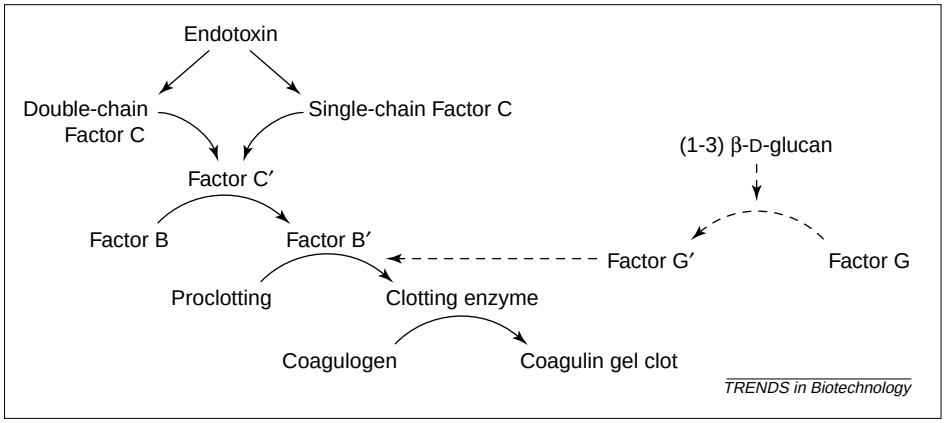

Fig. 1. The coagulation cascade reaction in the horseshoe crab amoebocyte lysate. Endotoxin activates both forms of Factor C. Single-chain Factor C exhibits a reversible activation reaction, which signifies a form of feedback regulation in the coagulation cascade[3]. Double-chain Factor C follows a path previously described for *Tachypleus tridentatus* amoebocyte lysate[5]. The pathway with dotted arrows is an endotoxin-independent alternate mechanism, in which Factor G is activated by (1-3) β-D-glucan[52].

During Gram negative bacterial sepsis[1], the pyrogenic endotoxin causes excessive release of inflammatory cytokines, leading to multiple organ failure and death. The ubiquity and indomitable nature of endotoxin has been a major challenge to the pharmaceutical and medical industries. Thus, reliable endotoxin diagnostics and therapeutics are urgently sought after. Endotoxin-induced coagulation cascade of the limulus amoebocyte lysate (LAL) represents a powerful defense used by horseshoe crabs against bacterial infection and the enzymatic components of the coagulation cascade (Fig. 1) have been well characterized[2–5], Factor C being the endotoxin-sensitive serine protease that initiates this coagulation cascade[3,5].

Owing to its extreme sensitivity to endotoxin, LAL has been used widely in the detection of endotoxin in pharmaceuticals, surgical implants, water and food (Table 1). The LAL test can detect femtogramme levels of endotoxin[6]. However, the following drawbacks have prompted an urgent need for an alternate source of LAL: (1) lack of specificity owing to LAL-reactive materials and fungal contaminant, [(1-3) β-D-glucan]; (2) batch-to-batch fluctuation in the sensitivity of commercial lysate to endotoxin; and (3) a dwindling population of horseshoe crabs (the source of LAL; Ref. 7). Recently, genetic engineering of an endotoxin-sensitive LAL protein, Factor C (Ref. 8) in *Escherichia coli*[9] yeast[10–12] and mammalian cells[13] yielded recombinant Factor C (rFC) capable of binding endotoxin[11,13]. Further cloning, manipulation and expression in a baculoviral system produced an rFC with remarkable enzymatic sensitivity to endotoxin at 0.001 EU ml^{-1} (Ref. 14).

This article leads from the initial discovery of LAL to 25 years of detection of endotoxin, problems associated with the use of LAL for quality assurance, and finally, to the cloning and expression of a biologically functional rFC. Because it is enzymatically activated by endotoxin, the rFC forms a convenient, perpectual, economical and standardized source of recombinant LAL for the detection of bacterial pyrogenic endotoxin.

From discovery to diagnostic

The initial discovery by Bang[15] that Gram negative bacterial endotoxin causes limulus blood to clot, followed by formulation of the first LAL (Ref. 16), have led to the Food and Drug Administration (FDA)-approval[17] and commercialisation[18] of LAL for testing endotoxin. The effects of bacterial endotoxins on humans range from fever to shock and death[19,20]. This underscores the importance of a reliable endotoxin test before any damage is caused. Even if there were a fully reliable endotoxin assay, there is no assurance that it would be helpful in providing effective clinical intervention based on the current lack of clinical success with anti-endotoxin reagents. Nevertheless, the LAL test has been used widely for the quality assurance of many products.

Some examples of the applications of the LAL test for endotoxin in pharmaceuticals, parenteral fluids, medical devices, water and food are presented in Table 1 (Refs 21–40). LAL has thus superceded the US Pharmacopiea (USP)-approved rabbit pyrogen test[41] the latter is time consuming, expensive and often subjective. The LAL assay is based on

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Table 1. Widespread applications of LAL for detecting endotoxin

	Refs
• **Clinical applications**	
Gram negative bacteriuria	21,22
Gram negative bacterial meningitis	23
Gram negative bacteremia and endotoxemia	24–26
Gonococcal urethritis	27
Legionnaires disease	28
Typhoid fever	29
• **Parenteral products and medical devices**	
Radiopharmaceuticals	30
Parenteral drugs	31
Antibiotics and antitumor preparations	32
Influenza virus vaccine	33
Large volume parenterals	34
Packaging components	35
• **Other applications**	
Endotoxin in water	36
Endotoxin in milk	37
Bacterial biomass in the marine environment	38
Assessment of quality of ground beef	39
Assessment of quality of lean fish	40

endotoxin-induced coagulation reaction of the limulus amoebocyte lysate and, over the years, LAL-based quantitation of endotoxin became possible with the design of new methods such as chromogenic, colorimetric and turbidometric assays[42–44].

Drawbacks with LAL – differential endotoxin reactivities and lack of specificity

Despite various methods of extraction of the lysate, the traditional clot assay and the ensuing quantitation assays are plagued with variations in sensitivity and specificity[45]. Several investigators have questioned the specificity of the LAL reaction for endotoxin. Thrombin, thromboplastin and certain synthetic polynucleotides result in positive LAL tests[46]. Peptidoglycan from Gram positive bacteria[47], exotoxins from group A Streptococci[48], simple polysaccharides including yeast mannans and bacterial dextrans[49], and dithiols[50] activate LAL to give false positive results. Furthermore, interference from LAL-reactive materials (LAL-RMs, e.g. anti-LPS factor and cellulosic material[51]), either causes false positive or false negative results in some batches of LAL. Unlike endotoxin, the LAL-RMs were consistently negative in the USP-rabbit test and failed to induce an inflammatory response. Thus, the question remains as to why this material reacts with certain LAL preparations and not with others.

Perhaps by reference to the biochemical characteristics of the lysate itself, an insight might be gained. The isolation and characterization of the components of the lysate cascade have been well documented[3,5]. The main pathway is triggered by endotoxin-sensitive Factor C, which activates intermediate serine proteases in the coagulation event to cause gelation or cleaves chromogenic substrates. This main cascade is joined by an alternative pathway (Fig. 1) via Factor G that is activated by (1-3) β-D-glucan[52] a 'contaminant' of fungal origin, yielding a false positive result in the LAL assay. However, an anti-LPS factor[45] present in untreated LAL, captures LPS to give a false negative result. Thus, the phenomenon of differential LAL reactivity in the absence of pyrogenicity has been widely observed and presents a recurring problem to endotoxin testing.

Problems with sample and specimen preparation

Given that LAL is composed of a series of coagulation enzymes, pH and temperature have a crucial influence over its reactions. The chelating agent EDTA, was found to inhibit endotoxin-induced LAL reaction, hence the importance of divalent cations to the integrity of the reaction[53]. Thus, components of the test sample could contribute to interference with any of the steps in the coagulation cascade, therefore affecting the final result. Owing to the dual biochemical characteristics of the lipopolysaccharide (or endotoxin) in that its polysaccharide portion is hydrophilic and its lipid portion is hydrophobic, LPS monomers will aggregate such that the lipid portion is hidden from the aqueous solution. However, it is the lipid portion that activates the LAL reagent and constitutes the pathogenicity of LPS (Ref. 54). To expose LPS to the LAL reagent, BioWhittaker Inc. (Walkersville, MD, USA) produced a metallo-modified polyanionic-dispersing agent, Pyrosperse®, which increases the amount of detectable endotoxin in inhibitory samples[55].

Persistent procurement of LAL threatens the 'living fossil' to extinction

Despite all the advances in creating new hardware, software[56] and continuous validation[57] of novel methodology designs in endotoxin testing, the biotechnology and medical industries still rely on the horseshoe crab for the lysate. However, recent calls in the USA for conservation of this 400 million year old 'living fossil' and the near extinction of the Japanese horseshoe crab, *Tachypleus tridentatus*[7], have placed a toll on the continued harvesting of the crab for commercial exploitation. The limulus species have important roles in the ecology and marine life of the US Eastern seaboard from Maine to Florida. Therefore, it is timely for us to conserve this 'living fossil', and replace natural LAL with cloned Factor C (Ref. 8) for endotoxin-testing.

A novel 'recombinant LAL' based on Factor C

Based on rFC, modern and yet simple, rapid, specific and sensitive diagnostic tests for endotoxin have been developed. As a proenzyme, rFC becomes catalytically activated by trace levels of endotoxin. The resulting activated rFC hydrolyses a synthetic substrate to form a quantifiable product, which measures the level of endotoxin. A fluorimetric assay for endotoxin uses rFC zymogen, which, on activation by endotoxin, hydrolyses a fluorogenic substrate such as Boc-Val-Pro-Arg-MCA (Boc, butoxy-carbonyl; MCA, 7-amido-4-methylcoumarin). The fluorimetric product is measured at an excitation of 380 nm and an emission of 460 nm. Raising the amount of rFC from 10 μg to 80 μg, further improves the sensitivity of detection of endotoxin from 0.005 EU ml^{-1} to 0.001 EU ml^{-1} (EU ml^{-1} is endotoxin units ml^{-1}, Fig. 2, Ref. 58). A comparison of rFC with commercial LAL, under the same assay conditions shows rFC to have lower background reading and a more sensitive response to endotoxin (Fig. 3).

Alternatively, a colorimetric assay is established using Boc-Val-Pro-Arg-pNA (pNA, *p*-nitroanilide) as chromogenic substrate that is hydrolysed by endotoxin-activated rFC to yield a measurable colorimetric product. Gel-filtration-purified rFC caused a drastic increase in the sensitivity of detection of endotoxin (Fig. 4; Ref. 58).

Further demonstration that rFC is capable of detecting endotoxin in a solid phase[58] is depicted in Fig. 5, which shows

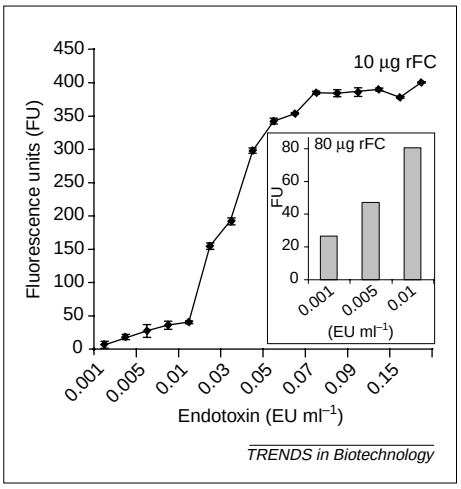

Fig. 2. Fluorimetric assay of endotoxin using 10 μg of gel filtration-purified rFC, which was reacted with endotoxin ranging from 0.001 to 0.1 EU ml^{-1}. A fluorogenic substrate, Boc-Val-Pro-Arg-MCA (see text) is cleaved by endotoxin-activated rFC, releasing fluorimetric product which is read at an excitation of 380nm and an emission of 460nm. Below 0.01 EU ml^{-1}, the endotoxin-induced enzymatic activity of rFC is biphasic, indicating co-operativity of endotoxin binding by multiple sites in the rFC molecule[61]. The inset shows that a sensitivity of 0.001 EU ml^{-1} was achieved using 80 μg of gel-filtration-purified rFC (Ref. 58). Results are the means ± SD of three independent experiments.

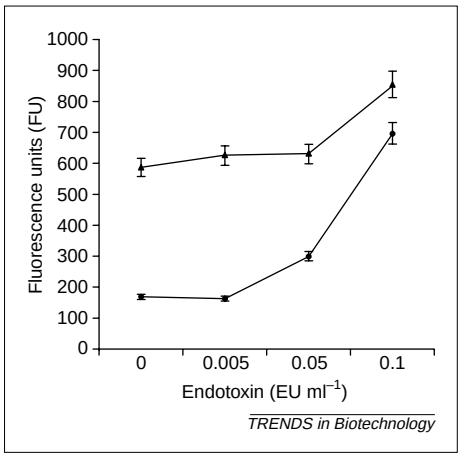

Fig. 3. Comparison of sensitivity of rFC and LAL to endotoxin[58]. Endotoxin standards (BioWhittaker Inc., USA) ranging from 0.005 to 0.1 EU ml^{-1} were used in the fluorimetric assay. 10 μg of gel filtration-purified rFC was used. Results are the means ± SD of three independent experiments. (Circles, rFC; triangles, LAL.)

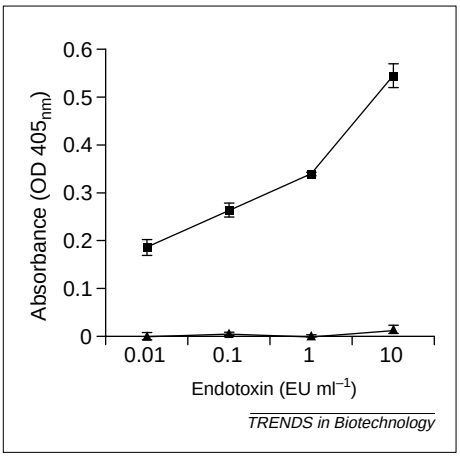

Fig. 4. In colorimetric assay, gel-filtration-purified rFC drastically improved the sensitivity of rFC to endotoxin compared with the concentrated crude rFC. Only 40 μg of gel-filtration-purified rFC was required to achieve this drastic increase in sensitivity compared with using 100 μg of the crude rFC (Ref. 58). Results are the means ± SD of three independent experiments. (Triangles, 100 μg concentrated crude rFC; squares, 40 μg gel-filtration-purified rFC.)

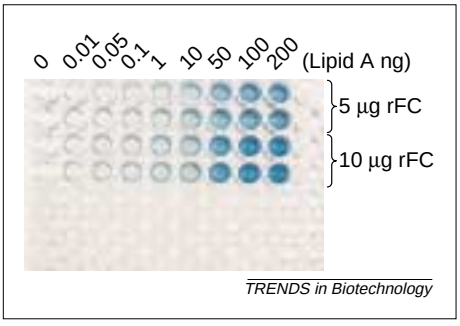

Fig. 5. ELISA-based detection of lipid A immobilized on a microtitre plate. Increasing intensity of colour development (at OD$_{405nm}$) of the enzymatically-hydrolysed product is seen from 0.01 to 200 ng lipid A (Ref. 58).

that using an ELISA-based assay, lipid A (the bioactive pathogenic moiety of endotoxin) immobilized on microtitre plate captures rFC, which binds anti-rFC antibody to interact with a secondary antibody conjugated with horseradish peroxidase. The captured secondary antibody then hydrolyses a chromogenic substrate [ABTS: 2,2′-azino-di-(3-ethylbenzthiazoline sulfonate)] yielding a coloured product, which corresponds to the presence of endotoxin.

A new method in pyrogen testing – genetically-engineered Factor C

The endotoxin test has a large market in drug companies that use LAL to detect endotoxin contamination in injectable products. Probably every major pharmaceutical company uses it for process monitoring, quality control and validation. Medical device firms also use the test to ensure that catheters, pacemakers and other invasive devices are endotoxin-free. In gene therapy, in which isolated plasmid DNAs are used as therapeutics, international regulatory agencies are bound to set stringent guidelines with regard to endotoxin levels[59]. With such great impact on the future therapeutic and diagnostic approaches, and the growing need for removal of endotoxins, it will be crucial to develop a sensitive, rapid and quantitative test for endotoxin using reliable and perpectual source of supply such as rFC. This new biotechnological innovation offers great promise in addressing several key issues in standardization of specificity and sensitivity, while being environmentally friendly. Also, the accumulated understanding of the molecular basis of interaction between endotoxin molecules and multiple endotoxin-binding sites of Factor C (Refs 60,61) will enable these researchers to deliver the much needed advancement in antimicrobial therapeutics.

This article describes, for the first time, a genetically engineered rFC (Ref. 14). The biologically functional rFC acts as a biosensor for endotoxin. The rFC remains a zymogen until it encounters trace levels of endotoxin, when it unequivocally exhibits full enzymatic activity, indicating the presence of endotoxin in a test sample. New microfluorimetric or microcolorimetric assays[58] allow high-throughput screens. The ELISA test indicates that rFC is capable of detecting immobilized lipid A and hence, offers a solid phase assay for endotoxin.

Conclusion

Despite problems associated with the lack of specificity of LAL, the biotechnological, pharmaceutical and medical industries have, over the past 25 years, relied on LAL for quality assurance of molecular biologicals, pharmaceuticals and medical devices. Although LAL is specific to endotoxin, many non-pyrogenic substances interfere with the assay. Furthermore, recent calls to protect the endangered population of horseshoe crabs have exacerbated these problems resulting in an urgent need to find a reliable replacement for LAL. Because it has multiple endotoxin-binding sites contained in each molecule[61], rFC amplifies the extreme sensitivity and specificity for quantitation of endotoxin and is therefore the appropriate candidate to replace LAL. The advent of rFC is timely because it will undoubtedly obviate the need to harvest horseshoe crabs and hence help to conserve this 'living fossil'.

Acknowledgements

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Meeting Report

Achieving compliance for biologics

Stephen Brown

The Validation of Biotech Manufacturing to Achieve Compliance conference was organized by Vision In Business and was held 30 and 31 January 2001 in Brussels, Belgium.

The manufacture of biologics (protein therapeutics) and associated topics have become popular subjects for conferences during the past few years. This has arisen partly owing to the greater awareness of the domain and the increasing number of products in this category but also because, within this diverse subject, there are real problems to overcome. Biologics cover a range of biologically derived medical products, often referred to as 'biotech products', and include blood derivatives, vaccines, recombinant proteins and gene therapy vectors. These products can be based on naturally occurring substances or microorganisms, or sourced from genetically modified organisms (GMOs). The processes used in biologics manufacture vary from those that are tried and trusted to 'hi-tech' approaches. Nevertheless, biologics all have one thing in common: regulatory compliance. This two-day conference examined the issues involved and roughly grouped the subjects into regulatory compliance, project management, process and analytical validations.

Regulatory issues

Regulatory issues occupied a major part of the conference. Martinus de Jonge (DSM Biologics, Groningen, The Netherlands) defined the requirements for biotechnology products manufacturing in terms of applicable laws based on European Directive 91/356/EEC and the US Code of Federal Regulations (21 CFR parts 210 and 211). It was shown how these enshrine quality assurance through current Good Manufacturing Practices (cGMPs). The applicability of the International Conference on Harmonization (ICH) GMP guide for Active Pharmaceutical Ingredients (APIs) was also reviewed extensively, particularly with regard to validation and qualification. A cost control perspective was also given. Trevor Deeks (Fluor Daniel, UK) pushed validation issues further, providing a stepwise overview of what is required when setting up a biotechnology facility.

An overview of the Committee for Proprietary Medicinal Products (CPMP) guidelines on comparability of medicinal products that contain biotechnology-derived proteins as active substances was presented by Pierrette Zorzi-Morre (French Medicines Agency, St Davies, France). These guidelines should be completed during the next six months; its aim is to analyse changes introduced into a process by the manufacturer, using a step-by-step multidisciplinary approach that will embrace the type of change, the complexity of the molecule and the quality implications. Crawford Brown (Eden Biopharm, Ellesmore Port, UK) covered process improvements and efficiency gains during product development and change control issues. During drug development, changes must be handled carefully; small changes upstream can cause major changes downstream. Additionally, in terms of what is possible, pragmatism is necessary. There are differences between the big pharmaceutical companies ('big pharma') and the small companies ('start-ups'), which don't have the same budgets or resources.

'…biologics all have one thing in common: regulatory compliance.'

Regulatory issues relating to cross contamination, the manipulation of GMOs and corresponding facility design issues was reviewed by Remi Gloeckler (Transgene, Strasbourg, France) and myself. Although there is broad international agreement concerning the definition of what makes a GMO and how they are classed and contained, differences between countries persist and these cannot be ignored. These points, and other containment requirements, must be taken into consideration when GMO-manufacturing facilities are designed. Regulatory issues were rounded-off with an excellent case study: Lynne Hill (Lonza Biologics, Slough, UK) reviewed how they have prepared and coordinated Food and Drug Administration (FDA) Team Biologics inspections. Team Biologics is now a combination of CBER and Office of Regulatory Assurance personnel and is the name used by the FDA for the external inspection process now used for biologics manufacturing facility audits. Inspections can last from a few days to a couple of weeks and concern a major team of investigators.

Project management

Project management issues were well represented. David Farrer (SR Pharma,

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