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Changing Global Perspectives on Horseshoe Crab Biology, Conservation and Management

Chapter 29

Biotechnology Efforts to Conserve Horseshoe Crabs Through the Development of Recombinant Factor C-Based Endotoxin Test

Peng Li, Bow Ho, and Jeak Ling Ding

Abstract During Gram-negative bacterial infection, endotoxin released from the outer membrane of the bacteria interacts with host sensor/receptor proteins to drive inflammatory responses. In severe persistent infection, septic shock ensues. Therefore endotoxin, which is ubiquitous, poses a threat to the biotechnology and healthcare industries. The quality assurance for injectable drugs and medical devices started with the slow, less efficient and expensive pyrogen test using rabbits. In 1970, the US Food and Drug Administration (FDA) approved the *Limulus* amoebocyte lysate (LAL) for testing endotoxin contamination. However, the LAL test requires the harvesting and bleeding of the horseshoe crab. Problems with the specificity of LAL to endotoxin and the potential for negative effects on horseshoe crab populations have called for an alternative reliable endotoxin test. Here, we review biotechnology efforts into recombinant Factor C (rFC), the endotoxin-inducible sensor protein in LAL, which establishes the foundation of a synthetic and rapid detection of endotoxin. Incidentally, in its “Guidance for Industry—pyrogen and endotoxin testings”, the US FDA has listed the rFC-based PyroGene® assay as an alternative endotoxin testing method. The industry has been slow to take the lead in using this highly sensitive test, which has been well-validated by the manufacturer.

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It is noteworthy that the rFC satisfies the typical analytical characteristics used in method validation (USP chapter <1225>). Thus, the sooner rFC can be accepted to replace the traditional LAL assay, the quicker and surer it will be to conserve horseshoe crabs, a threatened “living fossil”.

Keywords Conservation • *Limulus* amoebocyte lysate (LAL) test • Biotechnology • Recombinant Factor C (rFC) • Endotoxin/pyrogen assay

29.1 Introduction

In the process of infection and invasion in the host, the Gram-negative bacteria (GNB) release endotoxin, also known as lipopolysaccharide or exogenous biological pyrogen. The endotoxin is normally anchored on the bacterial outer membrane. The presence of endotoxin in the bloodstream of the host causes inflammation and can lead to sepsis. Endotoxin is recognized by receptors (e.g. CD14, TLR4) on the immune responsive cells, and this activates the endotoxin-signaling pathway. Endotoxin-stimulated human macrophages release inflammatory chemokines and cytokines. However, excessive inflammation causes septic shock, multiple organ failure and death (Bone 1996; McCormick et al. 2001). Thus, the ubiquitous endotoxin is a bane to the medical and healthcare industries as indicated by the wide application of the endotoxin test (Table 29.1). The US Food and Drug Administration (FDA) has set an endotoxin upper limit to 5.0 endotoxin units (EU)/kg body weight/hour per injection to ensure safe injectable drugs (Petsch and Anspach 2000). Hence, the biotechnology industry and pharmaceutical companies need a sensitive and specific endotoxin test to assure the endotoxin-free/endotoxin-low injectables and medical devices to pass US FDA guidelines prior to commercialization of parenteral products.

Table 29.1 The application of endotoxin detection assay in the biomedical industry

Application	Reference
Antibiotics product	Yamamoto et al. (2000)
Cardiac regeneration	Soncin et al. (2009)
Meningitis	Ichinohe et al. (1995)
Nanoparticles formulation	Dobrovolskaia et al. (2010)
Packaging components	Madsen et al. (2014)
Radiopharmaceuticals	Weatherman et al. (2013)
Sterile compound preparation	Cooper et al. (2010)
Urethritis	Prior and Spagna (1980)
Vaccine	Geier and Geier (2002)
Water contamination	Zhang et al. (2014)

29.2 Endotoxin: The Nemesis

Endotoxin or lipopolysaccharide (LPS) is also referred to as exogenous biological pyrogen because of its toxic and pyrogenic (fever-inducing) properties against the mammalian hosts. The membrane of an *E. coli* contains approximately 2×10^6 endotoxin molecules, constituting about 20 femtograms (Minabe et al. 1994). The endotoxin molecule comprises covalently linked fragments of the O-specific antigen, the outer and inner core oligosaccharides and lipid A (Ulmer et al. 2002).

Lipid A is the most structurally-conserved moiety of the endotoxin molecule (Freceer et al. 2000a, b). It is the bioactive center, as almost all endotoxin-induced biological reactions in the mammalian cells are lipid A-dependent. Direct evidence comes from studies of free native lipid A (Galanos et al. 1992) and synthetic lipid A (Takayama et al. 1983), both of which elicit full pyrogenic activity. Lipid A is made up of a phosphorylated β 1,6-linked D-glucosamine disaccharide that consists of variable numbers of asymmetrically placed amide or ester-linked acyl chains. This structure is the minimal necessity for the bioactivity of lipid A for it to induce cytokine production (Rietschel and Brade 1992) and inflammatory response. The distinctive structure of lipid A most likely suggests its important roles in the outer membrane of the bacteria as it confers resistance against host phospholipases, and hence, survival to the GNB. Endotoxins are uniquely thermo-stable and fairly insensitive to pH variations. The removal of endotoxin requires treatment at 200 °C for more than 2 h, or short durations in high concentrations of acids or bases, such as in pH 1.0 citric acid for 3 min (Minabe et al. 1994). Endotoxin is ubiquitous and indomitable in nature, existing in water, soil and the human gut, which hosts a wide variety of microbial commensals, including GNB. However, intravenous introduction of the endotoxin triggers a sequence of medical problems in the host. Thus, understanding the chemistry of endotoxin and defining its bioactive domain (Goh et al. 2002a, b; Freceer et al. 2004) has aided the design and development of better and more effective diagnostics and potential therapeutics against endotoxin.

29.3 Horseshoe Crab, a “Living Fossil”: Nature’s Gift/Foe Against Pyrogen?

The horseshoe crab, commonly referred to as *Limulus*, has been considered a “living fossil.” It belongs to an ancient protostome in the order, Xiphosura. Studies on the horseshoe crab have enlightened us on its evolutionary biology and palaeontology. There are four types of horseshoe crabs in the world: *Limulus polyphemus* (in the eastern coast of North and Central America); *Tachypleus tridentatus* (in East Asia) and *Tachypleus gigas* and *Carcinoscorpius rotundicauda* in Southeast Asia. These four extant species differ in morphology, size and habitat, especially, that of the *C. rotundicauda*—the smallest member, which thrives in estuarine swamps, while the larger *Tachypleus* and *Limulus* inhabit the seas.

Invertebrates offer an ideal model to study innate immunity in isolation since they lack adaptive immunity. Compared with other invertebrate models, the horseshoe crab has unique advantages. Firstly, it harbors a powerful innate immune system. As a mud-dwelling species, the *C. rotundicauda* co-habits with myriads of microbial pathogens, including Gram-negative and Gram-positive bacteria, and fungi. Throughout evolution, it has developed a plethora of formidable innate immune mechanisms, which have protected it from the extremely challenging microbes, and therefore it has conceivably co-evolved an excellent frontline immune defense system, thus providing a unique animal model for the study of innate immunity. Secondly, the horseshoe crab has a relatively large body size with substantial volume of tissues for biochemical and physiological studies. These studies are less feasible when using small invertebrates like the *Drosophila*. Thirdly, although the genomic DNA sequences of the horseshoe crab are still unavailable, the recent launch of the expressed sequence tag (EST) clusters (Ding et al. 2005) annotating frontline host defense, signaling, apoptosis and stress response genes of the horseshoe crab, has significantly facilitated the innate immune response study at the molecular level. During the last few decades, the horseshoe crab has been an ideal animal model for worldwide biotechnological research and innovations on the premise that alternative environmentally-friendly diagnostics and potential therapeutics were achieved in ways consistent with conservation of the species.

29.4 Factor C, an Endotoxin-Sensor in the Horseshoe Crab Blood

In an effort to produce LAL from *ex vivo* sources, researchers have attempted for several decades, but in vain, to aquaculture (Kropach 1979) and to establish a cell line from the horseshoe crab blood amoebocytes (Sanborn et al. 1959; Pearson and Woodland 1979; Ding et al. 1988). Parallel attempts were made to purify and characterize the LAL active enzymes to better understand how the blood clots in the presence of GNB (Muta et al. 1995; Ding et al. 2005). These efforts were made with a long-term goal of cloning the Factor C cDNA to synthesize an alternate pyrogen detection system (see Sect. 29.5).

The horseshoe crab relies on many of its innate immune proteins that are involved in different frontline defense mechanisms to stave off an infection (Ho et al. 1994; Yeo et al. 1996). Some examples include the complement cascade proteins like C3 (Ng et al. 2004; Tan et al. 2005; Zhu et al. 2005), melanization reaction in a phenoloxidase-induced production of anti-microbial compounds from a protein like hemocyanin (Nagai et al. 2001; Jiang et al. 2007; Du et al. 2010, 2011), lectins (Zhu et al. 2006; Li et al. 2007; Ng et al. 2007), antimicrobial peptide defense proteins, like tachyplesin (Nakamura et al. 1988a; Yeo et al. 1993), and an exceptionally sensitive blood coagulation cascade sensor protein, Factor C, that is triggered by picogram levels of endotoxin (Muta et al. 1991; Ding et al. 1993a).

The horseshoe crab hemolymph harbors mostly one kind of blood cells called amoebocytes, which are very sensitive to bacterial endotoxin. The amoebocytes release two types of granular components (large and small granules) into the plasma to participate in host defense via blood coagulation (Iwanaga and Lee 2005) and incapacitate the infecting microbe (Ding et al. 1988, 1993b). Figure 29.1 summarizes the defense proteins present in the large and small granules in the amoebocytes. Amongst all of these defense proteins, Factor C, which is present in the large granules, initiates the endotoxin-sensitive coagulation cascade and has been proposed to function in frontline defense against the invading GNB.

In the past decades, the coagulation cascade has been characterized (Iwanaga et al. 1994; Ding et al. 1995). This cascade consists of three serine protease zymogens: Factor C, Factor B, proclotting enzyme, and additionally, a clottable protein, coagulogen (Ho et al. 1993; Muta and Iwanaga 1996). As a biosensor of endotoxin, Factor C initiates the coagulation cascade reaction. Factor C in *C. rotundicauda* amoebocyte lysate (CAL) is capable of detecting trace levels of endotoxin (Navas et al. 1990). Ho (1983) further enhanced the sensitivity of endotoxin detection by spiking the test sample with known levels of endotoxin. Bacterial endotoxin induces the amoebocytes to aggregate and degranulate. Presumably, horseshoe crabs use the endotoxin-induced coagulation cascade as an important defense mechanism against the invasion of GNB (Armstrong and Rickles 1982). Thus, the horseshoe crab is heavily dependent on the coagulation cascade for its survival in the harsh environment. Nature seems to have provided the horseshoe crab with a powerful defense, which humans have exploited as a resource for LAL-endotoxin test (Levin et al. 1970) during the last >40 years.

Further search for other potential endotoxin-sensitive proteins in the horseshoe crab blood led to more research on additional serine protease cascades, which contribute to the fundamental biological processes in both the invertebrates and vertebrates.

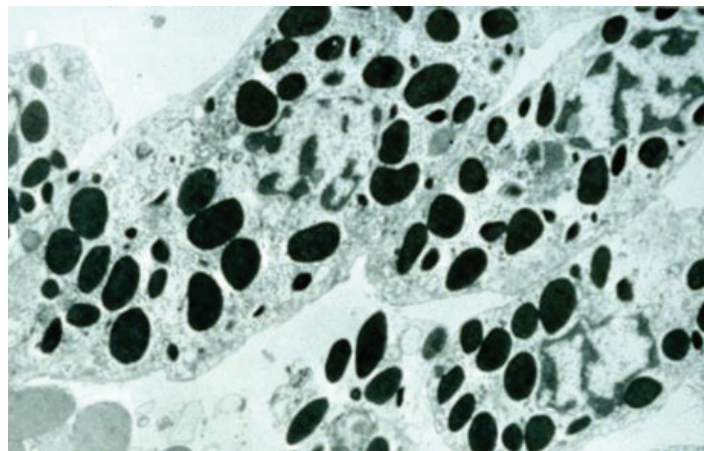


Fig. 29.1 The horseshoe crab amoebocytes. There are two types of electron dense granules, the large and small granules. Antimicrobial peptides are present in the small granules while Factor C and other coagulation cascade enzymes are present in large granules

Serine proteases play significant roles in signaling pathways involved in development, immunity, and hemostasis. However, no homologues of horseshoe crab Factor C have been found in mammals or insects besides the C-terminal serine protease domain sequence of the human α -thrombin, which bears 36.7 % similarity. A similarity between the Factor C-driven serine protease cascade and the *Drosophila* dorsoventral-determination serine protease cascade has been reported (Gay and Keith 1992)—the horseshoe crab clotting enzyme and the 36 *Drosophila* serine proteases including Snake and Easter, show significant sequence homology (Smith and DeLotto 1992; Ding et al. 1993a). Horseshoe crab Factor B, proclotting enzyme and coagulogen also exhibit a similar primary structure to the counterparts in *Drosophila* (Snake, Easter and Spätzle) (Wang et al. 2003), but there is no homologue of Factor C identified in the *Drosophila*. Thus, amongst the best studied invertebrate model organisms, only the horseshoe crab Factor C (a serine protease) appears to display unique endotoxin-binding properties. Is this an unfortunate endowment on the horseshoe crab, which is being exploited and endangered by humans?

29.5 Molecular Cloning of Factor C for Pyrogen Detection

Alternative: From LAL to rFC-Based PyroGene Test

Recently, genetic engineering of Factor C in bacteria (Roopashree et al. 1995, 1997a), yeast (Roopashree et al. 1996; Pui et al. 1997) insect cell line (Wang et al. 2001) and mammalian cells (Roopashree et al. 1997b) expressed recombinant Factor C (rFC) capable of agglutinating GNB and binding endotoxin. Subsequent cloning and molecular manipulations of the Factor C cDNA in the baculoviral-insect cell (*Spodoptera frugiperda*) system expressed rFC with an outstanding endotoxin-detection sensitivity at 0.001 EU/mL (Ding and Ho 2001). The rFC is a biologically functional synthetic biosensor for bacterial endotoxin. It remains as a zymogen until it binds to endotoxin, where it undergoes a conformational transformation to exhibit its full enzymatic activity, indicating the presence of endotoxin in a sample. Thus a rapid, sensitive one-step diagnostic assay for endotoxin has been developed (Fig. 29.2). In this assay, the stimulated rFC enzyme hydrolyses a synthetic substrate to produce a product, which quantifies the amount of endotoxin in the sample.

An ELISA-based colorimetric assay is formulated using Boc-Val-Pro-Arg-pNA (pNA, p-nitroanilide) as a chromogenic substrate that is hydrolysed by endotoxin-induced rFC to produce a measurable colorimetric product (Ding and Ho 2001). Alternatively, in a fluorimetric assay, the endotoxin-activated rFC hydrolyses a fluorogenic substrate such as Boc-Val-Pro-Arg-MCA (Boc, butoxy-carbonyl; MCA, 7-amido-4-methylcoumarin), yielding a fluorimetric product, measurable at an excitation at 380 nm and an emission at 460 nm.

Compared with the commercial LAL, which relies on all the coagulation cascade components to be functional to form a final reliable coagulation product, the rFC-based pyrogen assay has lower background and selective response to bacterial

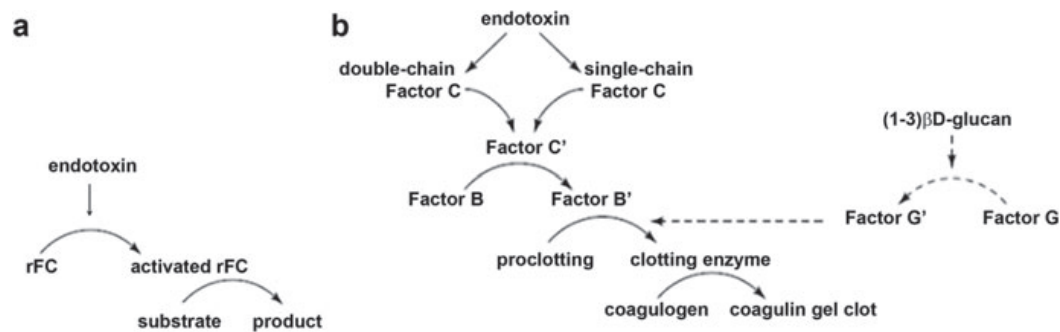


Fig. 29.2 Recombinant Factor C (rFC) pyrogen assay. (a) The novel single-step activation of rFC which hydrolyses a substrate to produce colored (colorimetric assay) result by colorimetric reaction, or fluorescence (fluorimetric assay) result by fluorimetric reaction, (b) The traditional Factor C-initiated coagulation cascade in the LAL assay (Adapted from (Ding and Ho 2001) with permission from Elsevier)

endotoxin (Ding and Ho 2001). In addition, the rFC assay, which involves only a single endotoxin-sensitive proenzyme, does not suffer from any potential false positive due to the fungal toxin, (1-3)β-D-glucan-activated Factor G pathway (see Fig. 29.2b). Furthermore, the microfluorimetric or microcolorimetric format integrated into the rFC assay has made it possible for rapid high-throughput screens of endotoxin in large quantities of samples (Ding and Ho 2001). Since 2000, the rFC clone has been outlicensed to Lonza Inc., which has incorporated it into a sensitive pyrogen-detection assay (PyroGene kit, Lonza Inc, <http://www.lonza.com/products-services/pharma-biotech/endotoxin-detection/endotoxin-detection-assays.aspx>).

29.6 How Can Biotechnology Circumvent the Need to Harvest and Bleed the Endangered Horseshoe Crab?

The rapid and specific detection of endotoxin is urgently needed for the development and production of clinical drugs and medical devices where state-of-the-art pyrogen-free facilities are required. It is necessary that every major pharmaceutical company employs efficient pyrogen testing for quality control and process endorsement. In biotechnology, where the purified plasmid DNAs from bacterial expressing-host cells, such as *E. coli* are used as diagnostics and therapeutics, the medical regulatory agencies are obligated to set strict rules to guard against endotoxin contamination. The biotechnological and pharmaceutical companies and healthcare industry have relied on LAL for quality assurance of parenteral products for more than 40 years, although there are problems related with the lack of specificity of the LAL assay. Recent programs to protect the dwindling population of *Limulus* have not eased these problems (Gauvry 2009; Ding and Ho 2010). In fact, harvesting of the horseshoe crab has intensified for both its blood and, in some areas, for cuisine (Liao and Li 2001; Berkson 2009). The rFC has offered several biotechnological applications—the horseshoe crab has contributed a reliable replacement for the

LAL-based pyrogen assay as well as a novel pyrogen-removal technology (Ding et al. 2001). Before it is too late, we must conserve this species without further harvesting and exploitation. Because of the presence of multiple endotoxin-binding sites on each molecule, the rFC is extremely sensitive to endotoxin and is, hence, the ideal replacement for the LAL assay. The replacement of the LAL assay with the rFC assay will avert the need to collect *Limulus* and thus, help to protect this endangered “living fossil”.

29.7 Perpetuity of the “Living Fossil” Depends on a Change in Human Mindset

Fifteen years passed between the FDA’s approval of the LAL test (1970; http://en.wikipedia.org/wiki/Limulus_amebocyte_lysat) and the start of basic research (mid-1980s) on the coagulation enzymes (Ding et al. 1988; Nakamura et al. 1988b), cloning and developing the rFC into a synthetic pyrogen test (Muta et al. 1991; Navas 1991; Roopashree et al. 1996; Ding et al. 1997; Pui et al. 1997; Tan et al. 2000; Ding and Ho 2001; Wang et al. 2002, 2003). In 1997, the environmentally-cognizant “BBC Tomorrow’s World” program drew the attention of the world to the genetic engineering of rFC, with the promise of an alternate and even better pyrogen test without ever having to harvest the horseshoe crab again! This year (2015) will mark the passing of another 15 years since the rFC clone was licensed (2000) to Lonza Inc. The rFC-based PyroGene waited for 12 years for the US FDA to list it as an alternative valid test method for endotoxin– “Guidance for Industry – Pyrogen and Endotoxins Testing: Questions and Answers”; www.fda.gov/drugs/guidance-compliancereulatoryinformation/guidances/ucm314718.htm.

Environmental concerns and conservation issues have loomed over the industry since the days of the rabbit pyrogen test, which predated the LAL test. There has been concern regarding survival of horseshoe crabs after they have been bled. Studies have shown that casualties may be as high as 30 % (Rudloe 1983; Thompson 1998; Walls and Berkson 2003). Lonza Inc (USA) and Hyglos Inc (Germany) reached an agreement to co-commercialize the rFC-based endotoxin test kits: PyroGene and Endozyme (<http://www.hyglos.de/en/news-events/news-events/news/article/press-release-lonza-and-hyglos-gmbh-reach-agreement-for-endotoxin-detection-patents.html>). One of the companies’ heads stated: “...we believe that finally, the sustainable animal-free alternative to LAL will be the future of endotoxin detection”. Various companies also market forms of LPS-removal kits. All of these businesses arising from research on the horseshoe crab are potentially good news for the “living fossil”; would this species now need to be further exploited for the industry? A concerted drive by: (1) the US and European Pharmacopoeias to approve, recognize, adopt, and mandate the use of the PyroGene and Endozyme assays worldwide to replace the LAL test; (2) the IUCN’s efforts and new government policies to prohibit the harvesting of horseshoe crabs (for blood or for food); and (3) a paradigm shift and a *bona fide* change in the mindset of ordinary

citizens and Pharmaceutical companies and Biotech industry towards a sincere sentiment for the conservation of the horseshoe crab will help ensure the perpetuity of this 500 million year-old “living fossil”.

29.8 Conclusion

The applications of the rFC in endotoxin detection have been supported by research work around the world. For many years, scientists have envisaged that research findings on the horseshoe crab innate immunity will benefit the biotechnology, pharmaceutical and human healthcare industries. There is no need for continuous harvesting from dwindling populations of horseshoe crab when there are readily available alternative tests that are more consistent and have a better endotoxin-detection limit compared to LAL.

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