Chapter 9
Endotoxin Detection – from *Limulus* Amebocyte Lysate to Recombinant Factor C

Jeak Ling Ding and Bow Ho

Abstract Gram negative bacterial endotoxin is a biological pyrogen that causes fever when introduced intravenously. The endotoxin, also known as lipopolysaccharide (LPS), is found in the outer membrane of Gram-negative bacteria. During Gram-negative sepsis, endotoxin stimulates host macrophages to release inflammatory cytokines. However, excessive inflammation causes multiple organ failure and death. Endotoxins, which are ubiquitous pathogenic molecules, are a bane to the pharmaceutical industry and healthcare community. Thus early and sensitive detection of endotoxin is crucial to prevent endotoxaemia. The limulus amebocyte lysate (LAL) has been widely used for ~30 years for the detection of endotoxin in the quality assurance of injectable drugs and medical devices. The LAL constitutes a cascade of serine proteases which are triggered by trace levels of endotoxin, culminating in a gel clot at the end of the reaction. The Factor C, which normally exists as a zymogen, is the primer of this coagulation cascade. In vivo, Factor C is the perfect biosensor, which alerts the horseshoe crab of the presence of a Gram-negative invader. The hemostatic end-point entraps the invader, killing it and limiting further infection. However, as an in vitro endotoxin detection tool, variations in the sensitivity and specificity of LAL to endotoxin, and the dwindling supply of horseshoe crabs are posing increasing challenges to the biotechnology industry. This has necessitated the innovation of an alternative test for endotoxin. Thus, Factor C became the obvious, albeit tricky target for the recombinant technology effort. This chapter documents the *backwater of mining* the natural blood lysate of the endangered species to the monumental effort of genetic engineering, to produce recombinant Factor C (rFC). The rFC is a 132 kDa molecule, which was produced as a proenzyme inducible by the presence of trace levels of endotoxin. The rFC forms the basis of the "PyroGene" kit, which is a novel micro-enzymatic endotoxin diagnostic assay for high-throughput screens of endotoxin. Using the rFC, Lonza Inc. has

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spawned the "PyroSense" which serves as checkpoints of the biotechnology production line. Thus, from cloning to commercial applications, the rFC has initiated a new era in endotoxin-testing for the quality assurance of biomedical products and for the healthcare industry, whilst sparing the endangered horseshoe crabs.

Keywords Endotoxin · Limulus amoebocyte lysate test · LAL · Molecular cloning · Pyrogene assay · Recombinant Factor C

Abbreviations

BPI	bactericidal/permeability-increasing protein
HDL	high density lipoprotein
GlcNAc	N-acetylglucosamine
GNB	Gram negative bacteria
KDO	2-keto-3-deoxy-D-mano-octonate
LAL	limulus amoebocyte lysate
LALF	limulus anti-LPS factor
LBP	LPS-binding protein
LDL	low density lipoprotein
LPS	lipopolysaccharide
rFC	recombinant Factor C
TNF-α	tumor necrosis factor alpha

9.1 General Introduction

Bacterial infections can be traced to exogenous and endogenous sources (Lent et al., 2001; Van Leeuwen et al., 1994; Lemaire et al., 1997). Gram negative bacteria (GNB) are ubiquitous and amongst the most challenging pathogens to the human host (Breithaupt, 1999; Zasloff, 2002; Boneca, 2005). Infection by GNB is the leading cause of sepsis (Bone, 1996; McCormick et al., 2001). It can cause excessive release of inflammatory cytokines, which can lead to multiple organ failure and death. The rising acquisition of multiple antibiotic resistance by superbugs is worsened by the lag in discovering and/or innovating new and more powerful antibiotics including antimicrobial peptides (Gradishar et al., 1995; Hancock and Chapple, 1999). There is a great urgency to circumvent the threat of new and more deadly species, which are capable of growing rapidly, even under challenging environmental conditions. The sporadic spread of Gram negative infection is exacerbated by the bioactive endotoxin (lipopolysaccharide, LPS) found on the outer membrane of the bacteria. The LPS and its juxtaposing anionic microbial phospholipids form the protective armour surrounding the bacterium. Upon death either by natural turnover events or killed by antibiotics during its invasion of the host, the Gram negative bacterium sheds its outer membrane releasing LPS. Thus, LPS is ubiquitous in nature, occurring in water, soil and the human gut which hosts commensals. Intravenous introduction of the LPS commences a string of medical problems instigated by the bioactive pharmacophore, the lipid A moiety of the LPS molecule. Thus, early and

highly sensitive detection of LPS is paramount to preventing and/or arresting the cascade of ill effects caused by LPS.

9.2 Gram-Negative Bacterial Membrane – A Wall of Fire

The Gram-negative bacterial membrane is a very well studied outer cell wall of a microbe. It represents the metabolite-based armour at the frontline of defense of the bacteria. Absent in eukaryotic hosts, and uniquely displayed on the outer membrane of the bacteria, these metabolites are collectively known as pathogen-associated molecular patterns (PAMPs). One such example is the LPS. For its grave ability to provoke pyrogenic action in an infected host, the GNB outer membrane indeed befits the description, "a wall of fire", as it is also needed as a fortress to protect the bacterium. Whether it is intact on the bacterium or released as cell wall fragments or as free LPS molecules in a mammalian host, the LPS wreaks inflammatory havoc leading to septic shock, and potentially, death to the invaded host-victim if not controlled on time. Despite extensive research on Gram negative septicaemia and efforts to develop antibiotics, infection by GNB is still a leading cause of sepsis, accounting for 45–60% of sepsis caused by bacterial infection (Bone, 1996; McCormick et al., 2001). The LPS stimulates the host's macrophages to release inflammatory cytokines, causing inflammation, which alerts the host of pathogen invasion. However, persistent exposure to LPS and excessive inflammation causes septic shock. Subsequently, multiple organ failure ensues and becomes the main clinical problem and cause of mortality (Brady and Otto, 2001). The patient can be rapidly killed by septic shock even before the bacteria could cause any direct harm (Ruiter et al., 1981). A multicentre observational cohort study had projected an estimated 751,000 cases of sepsis per annum in the United States alone (Chaby, 1999; Angus and Wax, 2001), which is comparable to that attributed to AIDS. In a small and developed country like Singapore with a population of 5 million, septicaemia is the 10th principal cause of death, with approximately one hundred casualties each year (http://www/moh.gov.sg/corp/publications/statistics/principal.do).

9.3 Lipopolysaccharide: A Mediator of Septic Shock – Pathophysiological Properties

The cascade of pathological outcomes and potential mortality triggered by LPS has drawn much research attention on the chemical structure-activity of the LPS molecule in order to help understand the molecular biology of Gram negative septicaemia. The LPS is also referred to as endotoxin because of its pyrogenic properties (fever causing) in human and other mammalian hosts. Introduced in the 19th century, this terminology, pyrogen, describes a component of GNB responsible for the pathophysiological phenomena associated with infection by GNB. Synthesized by bacteria as diverse as those responsible for cholera, whooping cough, plague and nitrogen fixation, a community's fear of endotoxin continues to attract wide interest by virtue of its role in bacterial infection and sepsis. Anchored on the outer cell

wall of the GNB (Ulmer et al., 2002, Brandenburg and Wiese, 2004), the LPS is an essential component of virtually all GNBs.

Unlike other toxic bacterial products that confer survival advantages but are otherwise non-essential, the LPS is presumably essential in all GNBs. The inability of GNB mutants to synthesise the minimally required LPS structure during the assembly of the outer membrane is a lethal event leading to the non-proliferation of the bacterium (Raetz, 1993). In contrast to most bacterial toxins, which directly injure eukaryotic cells by disrupting vital host cell functions, the LPS exerts its harmful effects more obnoxiously and indirectly by eliciting an exaggerated response of the host immune system (Corriveau and Danner, 1993; Karima et al., 1999). The host's innate immune response to LPS is beneficial in the event of a GNB infection as it creates a hostile environment for the invading bacteria. In the event that the innate immune system over-reacts, as in the case of overwhelming bacteraemia, the overproduction and systemic release of potent host-derived mediators such as proinflammatory cytokines, nitric oxide and eicosanoids may initiate a cascade of events which culminate in pyrogenic effect, shock, organ failure and death (Karima et al., 1999). The effects exerted by LPS are further complicated in vivo as LPS also stimulates the release of anti-inflammatory mediators such as transforming growth factor β, TGF-β (Dinarello, 1991). Thus, the phenomenon of endotoxaemia may actually represent conditions where there are imbalances between the proinflammatory and anti-inflammatory effects of LPS. The mammalian response to LPS is a complex and highly regulated process. It relies on both the humoral and cell membrane-bound recognition receptors, for example, TLR4 and CD14, which specifically interact with the lipid A moiety of LPS (Wright, 1991).

9.4 The Structure of LPS

Research on the LPS structure has focused on those derived from the enterobacteria (Takayama et al., 1983; Rietschel et al., 1996). Generally, the LPS is a structurally heterogenous, extremely resilient, indomitable and ubiquitous chemical molecule. The Gram-negative bacterium has been endowed with such a uniquely thermostable shield, which is also fairly insensitive to pH changes. Destruction of the endotoxin requires baking at 200°C for two hours or more, or shorter durations in the presence of high concentrations of acids or bases, for example in citric acid, pH 1.0 for 3 min. The envelope of a single *E. coli* is estimated to contain 2×10^6 LPS molecules, constituting about 20 femtograms (Minabe et al., 1994). LPS molecules are of great compositional and structural diversity, and yet, they are constructed according to a common architectural principle (Fig. 9.1). The LPS has a tripartite structure (Fig. 9.1) comprising three covalently linked domains: the O-specific chain, the core oligosaccharide and lipid A.

9.4.1 The O-Specific Chain

The O-specific chain is made up of a chain of repeating oligosaccharides of 3–8 units, which are specific to bacterial strains (Westphal et al., 1983; Raetz, 1990).

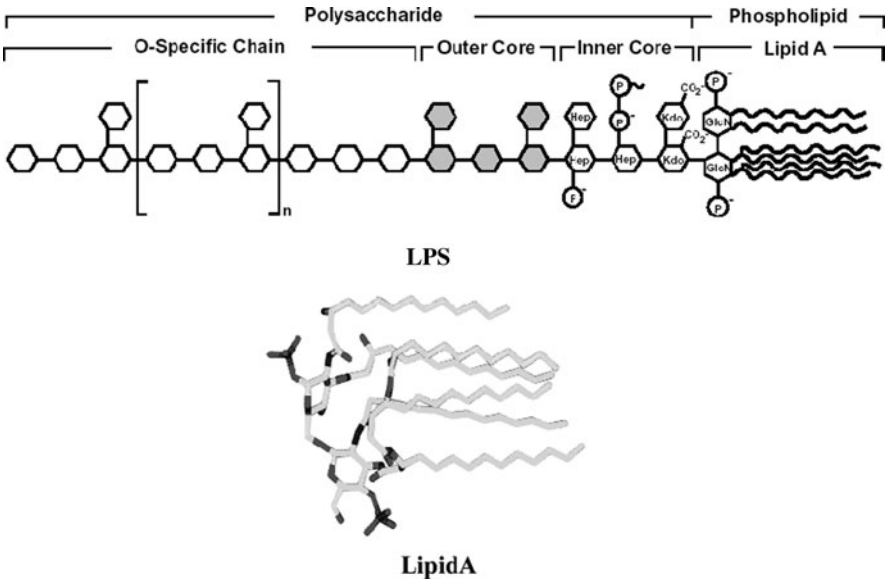

Fig. 9.1 The structure of lipopolysaccharide, LPS. LPS consists of an O-specific antigen, a core oligosaccharide and the lipid A moiety. The core oligosaccharide, which varies from one bacterial species to another, is made up of outer and inner sugar regions. Lipid A virtually always includes two glucosamine residues modified by phosphates and a variable number of fatty acid chains (Frecer et al., 2000). The LPS structure was kindly contributed by Professor Helmut Brade (Research Center Borstel, Leibniz-Center for Medicine and Biosciences, Medical and Biochemical Mikrobiology Parkallee 22, D-23845 Borstel, Germany)

This domain determines the serological identity of the respective bacterium (Petsch and Anspach, 2000). Therefore, considerable structural diversity is noted amongst the O-specific antigen chain structures of LPS from different GNBs. Furthermore, the O-chain polysaccharides are the major immune-reactive domain of the LPS molecule, hence, this domain is often referred to as the O-specific antigen. It is responsible for defining the serotypic specificity of an individual GNB strain. Although the O-specific chain of LPS confers survival advantages, such as prevention of serum-mediated lysis of the bacterium, it is reported to be unessential for bacterial growth or survival (Raetz et al., 1988). This is reasonably logical since it would be consistent with the manner in which the GNBs are able to adapt to different modes of culture (agar plate or LB broth), and survive against challenges of the changing environment.

9.4.2 The Core Oligosaccharide Domain

The core oligosaccharide is a complex, non-repeating oligosaccharide that contains a unique inner sugar, KDO (2-keto-3-deoxy-D-mano-octonate)-heptose region which links the core oligosaccharide to the lipid A moiety. The core oligosaccharide is more conserved than the highly variable O-specific chain (Brade et al., 1988).

It is made up primarily of glucose, galactose and N-acetylglucosamine (GlcNAc) (Jansson et al., 1981; Rick, 1987). The hexose molecules in the outer core are more variable in structure than the inner core. The KDO sugar is linked directly to the lipid A moiety (Rietschel and Brade, 1992). Since it is buried within the LPS molecule, it was believed to be sterically-hindered from interaction with the host immune system (Giglioti and Shenep, 1985; Pollack et al., 1989; Heumann et al., 1991). It has been established that at least part of the core oligosaccharide, viz, the KDO-containing part is essential for bacterial viability (Raetz et al., 1991).

9.4.3 The Lipid A

The lipid A which is the minimum structure of LPS capable of sustaining bacterial growth and survival, is the most conserved moiety of the LPS molecules derived from diverse strains of GNB. The lipid A moiety, attached to two or three KDO residues (Lynn, 1998), acts as the membrane anchor of the LPS molecule. Lipid A is considered to be the bioactive centre of the LPS as virtually all LPS-induced biological responses in the host cells are lipid A-dependent. The synthetic lipid A (Galanos et al., 1992) was shown to display full endotoxic activity compared to the free lipid A cleaved from the native LPS molecule (Takayama et al., 1983), thus strongly suggesting the pathophysiological significance of the lipid A moiety. The lipid A is composed of a phosphorylated β1,6-linked D-glucosamine disaccharide (Frecer et al., 2000a, b) that carries variable numbers of asymmetrically placed amide or ester-linked acyl chains. This structure is the minimal requirement for the cytokine inducing capacity of lipid A (Rietschel and Brade, 1992). The unique structure of lipid A most likely reflects its important roles in the outer membrane assembly and functions, and it ensures resistance to phospholipases.

The structures of GNB lipid A such as those of *Escherichia coli* and *Salmonella typhimurium* have been elucidated (Takayama et al., 1983). The classical *sn*-1,2-diacylglycerol moiety of membrane phospholipids is replaced by a 2,3-diacylglucosamine unit in the lipid A moiety (Raetz, 1990). The acyl chains that are attached to the glucosamine backbone of lipid A differ from those attached to the membrane phospholipids in that they are 2–6 carbon atoms shorter and contain an (R)-3-hydroxyl substituent. The unique structure of lipid A presumably reflects its specific roles in the outer membrane assembly and function, and it ensures resistance to phosholipases. The lipid A of *E. coli* and *S. typhimurium* are β(1,6)-linked disaccharides of D-glucosamine that are acylated with (R)-3-hydroxytetradecanoic acid at positions 2, 2', 3, and 3', and phosphorylated at positions 1 and 4'. The two (R)-3-hydroxyl-acyl groups at positions 2' and 3' of the nonreducing glucosamine are further esterified with dodecanoic acid and tetradecanoic acid. The envelope of a single *E. coli* cell contains approximately 2×10^6 lipid A residues and approximately 2×10^7 glycerophospholipids, quantities consistent with the existence of one monolayer of lipid A and three monolayers of glycerophospholipids.

Since the structural determination of lipid A has focused on those of *E. coli* and *S. typhimurium* and that similar lipid A structures are reported to be present in the Enterobacteriaceae family, their lipid A structures are taken to be the prototype of

lipid A structures, viz, "usual" lipid A (Mayer and Weckesser, 1984). However, intrinsic heterogeneity and species- and strain- specific modifications of lipid A do occur. Variations in the phosphorylation pattern, acylation pattern and the fatty acid chain length of lipid A (Kawata et al., 1999) in other GNB species as opposed to those of *E. coli* and *S. typhimurium* lipid A could lead to a change in the endotoxic potency of the molecule. Many of the variant, "unusual" lipid A were observed to be non-endotoxic, even though the exact prerequisites of endotoxic activity remain unclear. For the full expression of endotoxic activities, the structural requirements of lipid A allow for only small variations from those of *E. coli* or *S. typhimurium*, viz, a β(1,6)-linked D-glucosamine disaccharide carrying two negatively charged phosphates and six saturated fatty acids in a defined asymmetrical 4/2 distribution.

9.5 Plasma LPS-Binding Proteins Protect and Provoke Septic Shock – The Achilles Heel?

As a pathogen-associated molecular pattern (PAMP) molecule of the GNB, the LPS is a key virulence factor. During infection, the host's innate immune defenses immediately respond to the GNB invasion via LPS-induced signal transduction pathways, finally resulting in inflammation and septic shock (Fig. 9.2). Upon infection, the bacteria release LPS into the bloodstream to trigger the innate immune system, which initiates a series of defenses against the invasive GNB via specific recognition mechanisms (Ng et al., 2007; Jiang et al., 2007; Le Saux et al., 2008; Zhang et al., 2009; Jiang et al., 2009).

Certain circulating proteins in the plasma of mammals appear to interact with LPS in ways that can inhibit or enhance the immunological response to LPS (Corriveau and Danner, 1993). Plasma lipopolyproteins such as low density lipoprotein (LDL) and high density lipoprotein (HDL), which are involved in the transport and metabolic regulation of triglycerides and cholesterol, can bind LPS, reduce its toxicity and promote its clearance from circulation through the hepatobiliary system (Harris et al., 1993; Read et al., 1993; Feingold et al., 1995; Chaby, 1999). Non-lipoprotein factors, such as LPS binding protein (LBP) and soluble CD14 (sCD14), discussed later in this section, appear to be important in facilitating LPS-lipoprotein interaction. In this regard, plasma lipoproteins form part of the innate immune defense system against LPS.

The LBP, a 60 kDa glycoprotein of hepatic origin acts as an opsonin by enhancing the interaction of LPS with phagocytes. LBP binds to LPS via the lipid A moiety (Schumann et al., 1990). The LBP-LPS complex subsequently interacts with CD14 (Wright et al., 1990), a 55 kDa glycophosphatidylinositol (GPI)-linked receptor protein that is found on the surface of macrophages, monocytes and neutrophils (Ziegler-Heitbrock and Ulevitch, 1993; Kim et al., 2005).

The LBP facilitates the binding of LPS or GNB to phagocytes (Tobias et al., 1988; Corriveau and Danner, 1993; Yu and Wright, 1996). The enhanced interaction between LPS and phagocytes causes a marked increase in cell activation. Addition of small amounts of LBP to cultured macrophages increases by 100-fold the ability of LPS to induce the production of tumor necrosis factor alpha (TNF-α), a potent

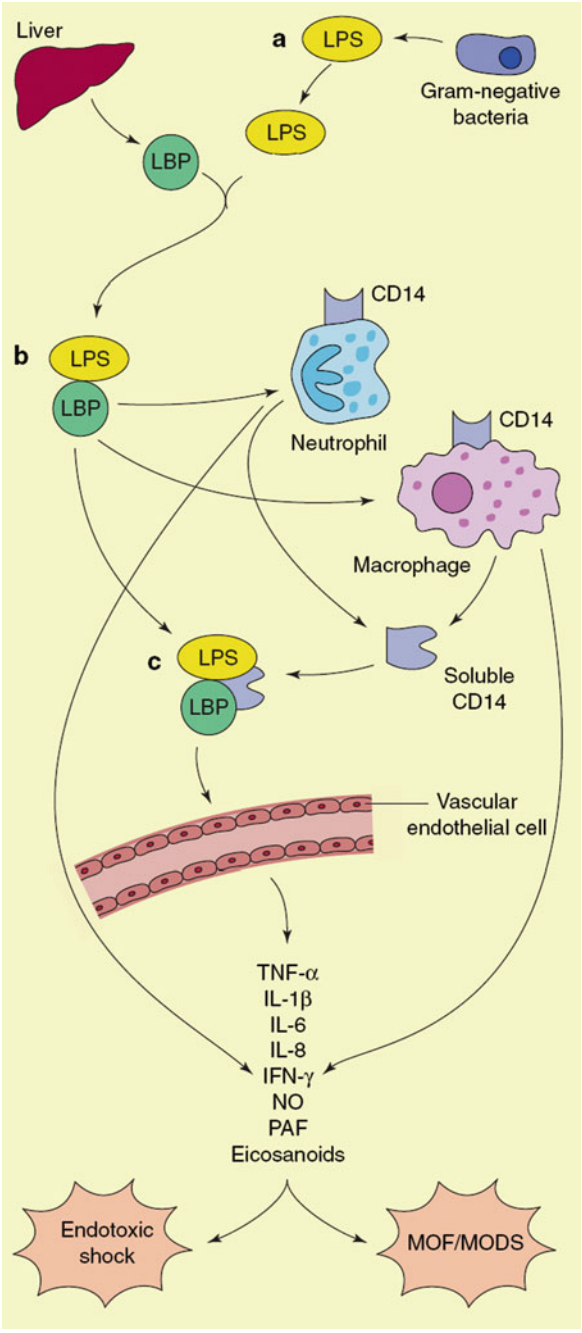

Fig. 9.2 The host cellular activation by lipopolysaccharide. (**a**) In the plasma, LPS is released from the Gram negative bacteria. (**b**) LPS-binding protein (LBP) transfers LPS to CD14 and facilitates the interactions of LPS with CD14 expressed on the surface of monocytes/macrophages or neutrophils. Endothelial cells and some other types of cells do not express CD14. (**c**) LPS stimulates these cells by binding soluble CD14. IFN-γ, interferon γ; IL-1, interleukin 1; MOF/MODS, multiple organ failure/multiple organ dysfunction syndrome; NO, nitric oxide; PAF, platelet-activating factor; TNF-α, tumor necrosis factor. Adapted from (Karima et al., 1999), with permission from Elsevier, Copyright Clearance Centre

proinflammatory cytokine. This phenomenon has been observed with different types and sources of LPS (Schumann et al., 1990). In acute phase response, the plasma LBP level can escalate 1000-fold, from a basal concentration of 0.5 g/ml to 50 g/ml, within 24 h, leading to a surge in cell activation. Besides LBP, septin (the product of a proteolytic cascade distinct from the coagulation and complement systems in the human plasma) binds LPS and mediates LPS recognition by phagocytes in a manner similar to LBP (Wright et al., 1992).

Bactericidal/permeability-increasing protein (BPI) is a 55 kDa inducible cationic protein of neutrophil origin that binds the lipid A moiety and neutralizes a variety of LPS. BPI is a boomerang-shaped molecule, comprising two domains located at the amino and carboxy termini that are structurally similar. These domains are hydrophobic and they bind lipid A (Beamer et al., 1997). Although BPI is thought to be primarily involved in the non-oxidative killing of ingested bacteria, evidence suggesting localization of BPI on the surface of neutrophils indicate that BPI is able to detoxify LPS in circulation (Weersink et al., 1992).

In the horseshoe crab, the LPS-binding proteins include the Limulus anti-LPS factor (LALF), which is a small basic protein of 101 amino acids that not only binds LPS but elicits a strong antibacterial effect on GNB (Morita et al., 1985b). Based on the sequence similarity and the crystal structure of LALF, Hoess et al. (1993) suggested that an exposed amphipathic loop on LALF, distinguished by an alternating series of positively charged and hydrophobic residues, represents an LPS-binding motif.

Another LPS sensor is the Toll-like receptor 4 (TLR4)-MD-2 complex, which is localized on the immune-responsive cell membrane (Yang et al., 2000). Protein kinases, such as p38 and JNK (Sweet and Hume, 1996) are triggered by LPS induction, leading to the activation of several transcription factors such as NF-κB, which in turn activates transcription of genes coding for numerous proinflammatory cytokines, tissue factors, adhesion molecules and inducible nitric oxide synthase. The overproduction of these potent mediators initiates a series of pathophysiological events that culminates in clinical manifestations of sepsis. Amongst the proinflammatory cytokines, TNF-α plays a critical role in the inflammatory response and is often regarded as a hallmark of LPS-induced inflammation (Tang et al., 2005). Following LPS challenge, the immunological cascade encompassing CD14, TLR4, MAPK and NF-κB (Guha and Mackman, 2001; Akira et al., 2006) is swiftly activated to sensitize the host to an LPS-induced uncontrolled acute inflammatory response that can result in septic shock, multiple organ failure/deaths, MOF/MODS (Fig. 9.2) (Li et al., 2006).

The LPS also interacts with the humoral immune system. It activates both the complement cascade which further fuels the inflammatory response, and the coagulation cascade which leads to disseminated intravascular coagulation that quickly depletes the clotting components in the blood leading to haemorrhage (Glauser et al., 1991). Therefore, at the initial stage of Gram negative infection, the binding of the LPS-receptors to LPS is required to trigger an inflammatory response, and yet, an immune over-reaction leads to septic shock and further downstream casualties. Therefore, LPS-binding proteins are analogous to an "Achilles heel", protecting and yet provoking inflammatory responses. Thus, recent studies are

directed towards the intervention of this early step with potential LPS-binding drugs to compete against binding of LPS-effectors, and to attenuate the consequential damage to the host. However, an even more upstream step is the detection of LPS in a parenteral product, which may circumvent the need to intervene LPS-intoxication. This might indeed be the best option towards prevention rather than resolution-after-contamination/infection, since the LPS molecule is extremely indomitable.

9.6 Overcoming the LPS Problem – The Horseshoe Crab, a Creature Small and Great

LPS is the best-studied biological pyrogen. Upon intravenous introduction, it causes fever/pyrogenic action. Owing to its ubiquity, the LPS has been a bane to the pharmaceutical and medical industries since parenteral preparations contaminated with trace levels of LPS (in the picogram levels), can elicit dramatic effects in the patients. Thus, reliable endotoxin diagnostics and therapeutics are urgently sought.

The horseshoe crab hemolymph contains mainly one type of blood cells called amoebocytes (Fig. 9.3), which are extremely sensitive to LPS. During a Gram negative infection, the amoebocytes release granular components into the plasma

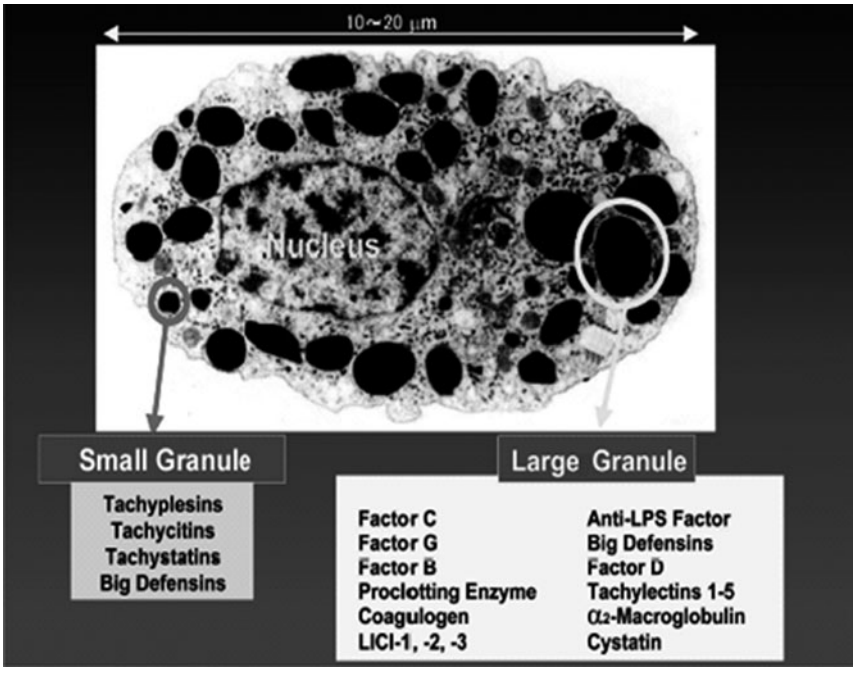

Fig. 9.3 The amoebocytes of horseshoe crab. Some small antimicrobial molecules like antimicrobial peptides that have been identified are in small granules, while Factor C and other defense molecules are localized in the large granules. Adapted from (Iwanaga and Lee, 2005), with permission from Editor-in-Chief of the Journal of Biochemistry and Molecular Biology

to participate in self-defense via blood coagulation (Iwanaga and Lee, 2005), which incapacitates the invading microbe. The amoebocytes contain two kinds of secretory granules, the large and small granules. Studies on these granules suggest that coagulation factors such as Factor C, are localized in the large granules whereas the antimicrobial peptides such as tachyplesin, are contained exclusively in the small granules (Iwanaga, 2002).

In the past decade, the molecular mechanisms of the coagulation cascade have been established (Navas et al., 1990; Ho et al., 1993; Ding et al., 1993a; Iwanaga et al., 1994; Ding et al., 1995). LPS from the GNB induces the amoebocytes to degranulate, thus initiating the blood coagulation cascade (Fig. 9.4), which is an important defense mechanism used by horseshoe crabs to kill and trap the invading GNB (Armstrong and Rickles, 1982; Ding et al., 1993b). This cascade is based on three serine protease zymogens – Factor C, Factor B, proclotting enzyme and one clottable protein, coagulogen (Muta and Iwanaga, 1996). Factor C, at the first step of the coagulation pathway, is sensitive to LPS. Interestingly, no homologues of Factor C have been found in the mammals, although the C-terminal serine protease domain has substantial similarities (36.7%) with human α-thrombin (Li et al., 2007). Thus, Factor C is a unique LPS-binding protein found only in the horseshoe crab.

As a "living fossil" which has survived for several hundred million years, the evolutionary success of the horseshoe crab attests to its strong innate immune defense ability, which it uses to thrive in microbiologically harsh habitats with abundant variety of disease-causing pathogens.

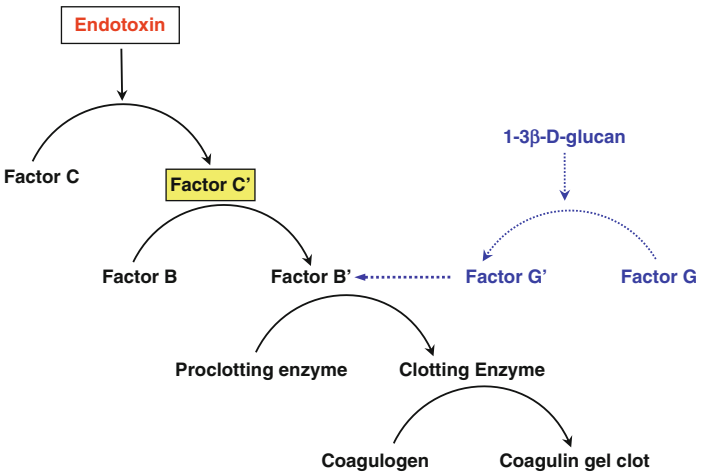

Fig. 9.4 The coagulation cascade in the horseshoe crab amoebocyte lysate. In the presence of LPS, the Factor C serine protease zymogen is autocatalytically activated to an active form, Factor C', which activates the proenzyme Factor B into Factor B'. This in turn activates proclotting to active clotting enzyme. Clotting enzyme then converts coagulogen into a coagulin gel clot, which traps the invading bacteria

The American horseshoe crab, *Limulus polyphemus* amoebocyte lysate (LAL) is well known and its coagulation cascade has been fully characterized and shown to defend the animal against the invasion of Gram-negative bacteria (Armstrong and Rickles, 1982). Owing to its extreme sensitivity to the bacterial endotoxin, LAL has been widely marketed for decades as a tool for detecting LPS in pharmaceuticals, parenterals and surgical implants, water and food (Levin et al., 1970; Novitsky, 1994). The LAL can detect femtogramme levels of LPS (Ho, 1983).

9.7 Drawbacks with LAL

Although the LAL test has been introduced since the 1970s, as a replacement for the rabbit pyrogen test for the quality assurance of parenterals and medical devices, it has suffered many severe setbacks. First, batch-to-batch and seasonal variations in LAL preparations cause differential sensitivity to LPS. Second, the lack of specificity for endotoxin is compounded by fungal contaminant, 1–3 β-D glucan (Fig. 9.4), which switches on the alternate coagulation pathway (Iwanaga et al., 1985), resulting in false positive test for pyrogen. Third, besides providing their newly laid eggs an energy boosting meal for migratory birds, the biomedical and economic importance of the horseshoe crab blood has caused the population of the American *Limulus polyphemus* to drop alarmingly. We describe below, some of the problems associated with the LAL test.

9.7.1 Differential Endotoxin Reactivities and Lack of Specificity

Despite years of intense efforts made with various methods of extraction of the amoebocyte lysate, the traditional clot assay and other quantitative assays were plagued with variations in sensitivity and specificity for LPS (Jorgensen and Smith, 1973). The specificity of the LAL assay for endotoxin is interfered by proteins and cofactors such as thrombin, thromboplastin, and certain synthetic polynucleotides, which can all give false positive results. On the other hand, peptidoglycan from the Gram positive bacteria, exotoxins from group A Streptococci (Brunson and Watson, 1976), simple polysaccharides including yeast mannans and bacterial dextrans and dithiols can also activate LAL to give a false positive or false negative result in some batches of LAL. As shown in the coagulation pathways (Fig. 9.4), the main pathway is triggered by endotoxin-sensitive Factor C, which activates intermediate serine proteases in the coagulation event to cause gelation in vivo, or to cleave a chromogenic or fluorogenic substrate in an in vitro reaction. However, an alternative cascade driven by the fungal toxin 1–3 β-D-glucan activates Factor G, which joins the main pathway and ultimately also leads to coagulin formation in vivo, and/or hydrolysis of the synthetic substrate in an in vitro assay, causing a false

positive result. Thus, fungal contaminant will give a false positive result, which may be interpreted as endotoxin-positive. This explains the phenomenon of differential LAL reactivity in the absence of pyrogenicity, which has been widely observed and presents a recurring problem to endotoxin testing.

9.7.2 Problems with Sample and Specimen Preparations

Since LAL contains a series of coagulation enzymes, the pH, temperature and ionic strength have a critical influence over the precise biochemical reactions to ensure an appropriate end-point, coagulin formation. Components of the test sample can interfere with any step in the coagulation cascade, thereby affecting the final result. EDTA was found to inhibit endotoxin-induced LAL reaction (Morita et al., 1985), thus indicating the importance of cationic balance in this reaction. The LPS monomers, which contain both hydrophilic sugar groups and hydrophobic lipid A portion, have a propensity to form heteropolymers /micelles of different sizes, with the lipid A portion hidden from the aqueous environment although it is the lipid A that is the endotoxic moiety of the LPS molecule. Attempts are being made to overcome this problem; BioWhittaker Inc. (now Lonza Inc.) and BioDTech Inc. have produced dispersing agents, which increase the amount of detectable endotoxin in inhibitory samples.

9.7.3 LAL Production Endangers the Horseshoe Crab

Despite all the advancements made with new hardware and software and novel design methodologies, the biotechnology and medical industries still rely heavily on the horseshoe crab blood lysate to pass quality assurance of injectables and medical devices. Over-harvesting of the horseshoe crab by the Biotechnology Industry and the food chain-induced loss of the horseshoe crab spawned eggs, due to feeding by migratory birds, have dramatically dwindled the horseshoe crab population into potential extinction (Widener and Barlow, 1999). Furthermore, due to urbanization, the Japanese horseshoe crab was pronounced endangered since 1973 (Sekiguchi and Nakamura, 1979) and much effort continues in re-spawning and conserving the species (Sekiguchi et al., 1982, 1988).

These drawbacks have prompted many researchers to look into long term alternatives like: (i) farming of horseshoe crabs for the procurement of blood (Kropach, 1979), (ii) tissue culture of amoebocytes from which LAL originates (Pearson and Woodland, 1979) and (iii) genetic engineering of lysate proteins such as Factor C (Muta et al., 1991; Ding et al., 1995), the first serine protease of the coagulation cascade which is enzymatically-activated by LPS. It was necessary to face the challenge of producing a genetically-engineered LAL to replace the conventional LAL test; one which is more reliable, sensitive and specific for LPS and without relying on harvesting blood from the endangered horseshoe crab.

9.8 Factor C: A Horseshoe Crab Serine Protease with Multiple High Affinity LPS-Binding Sites – LPS Detection and Prevention Strategies – Towards Non-LAL Based LPS Detection

After 2–3 decades of conventional pyrogen testing using the blood extract of the horseshoe crab, and the problems associated with LAL, hereon, we describe a breakthrough in genetic engineering (US Patent No: 5,712,144) and molecular expression of recombinant Factor C, which yields enzymatically active rFC that is activated by trace levels of LPS, with a remarkable sensitivity of 0.001 EU/ml. Thus, rFC serves as a novel, perpectual, environmentally-friendly and standardised source of "LAL" for sensitive and specific detection of LPS. Using rFC, a novel micro-assay (US Patent No: 6,645,724B1) was developed for high throughput screens of pyrogen in pharmaceuticals and parenterals. A US Biotech Company that is a major producer of LAL is commercialising the rFC as a novel endotoxin diagnostic that sets a new standard for pyrogen testing. Furthermore, being capable of binding both free and bound LPS / lipid A (the biologically potent moiety of LPS) with high affinity, the rFC has other potential applications, such as the removal of LPS from contaminated samples (US Patent No: 6,645,724), as well as for the development of novel endotoxin therapeutics and antimicrobials.

The initial discovery by Bang (1956) that Gram negative bacterial endotoxin causes limulus blood to clot, followed by the formulation of the first LAL (Levin et al., 1970), have led to the FDA-approval (Sullivan and Watson, 1978) and commercialization (Novitsky, 1984) of LAL for testing endotoxin. Since the mid-1970s, LAL has been used widely for the quality assurance of many products approved by FDA. Therefore, LAL has superceded the US Pharmacopioea (USA)-approved rabbit pyrogen test. The latter is time consuming, expensive and often subjective. Over the years, LAL-based quantitation of endotoxin became somewhat possible with the design of new methods such as chromogenic, colorimetric and turbidometric assays (Iwanaga et al., 1978), although still haunted by problems with either the sample to be tested or the LAL preparation itself. Being at the initial step of the coagulation cascade, Factor C functions as a very sensitive and specific biosensor of LPS, capable of detecting picogram to nanogram levels of LPS (Ho, 1983), hence it was a prudent step to genetically engineer Factor C to replace the LAL.

9.9 Genetic Engineering and Production of Recombinant Factor C (rFC) – Necessity Spawns Innovation: Cloning and Subcloning the Factor C cDNA into Bacterial, Yeast, Insect and Mammalian Cells

Factor C is the LPS-sensitive intracellular serine protease zymogen that initiates the coagulation cascade system. It exists as a single- and a double-chain form (Ding et al., 1993). While the catalytic site of the molecule is found in the light chain, the

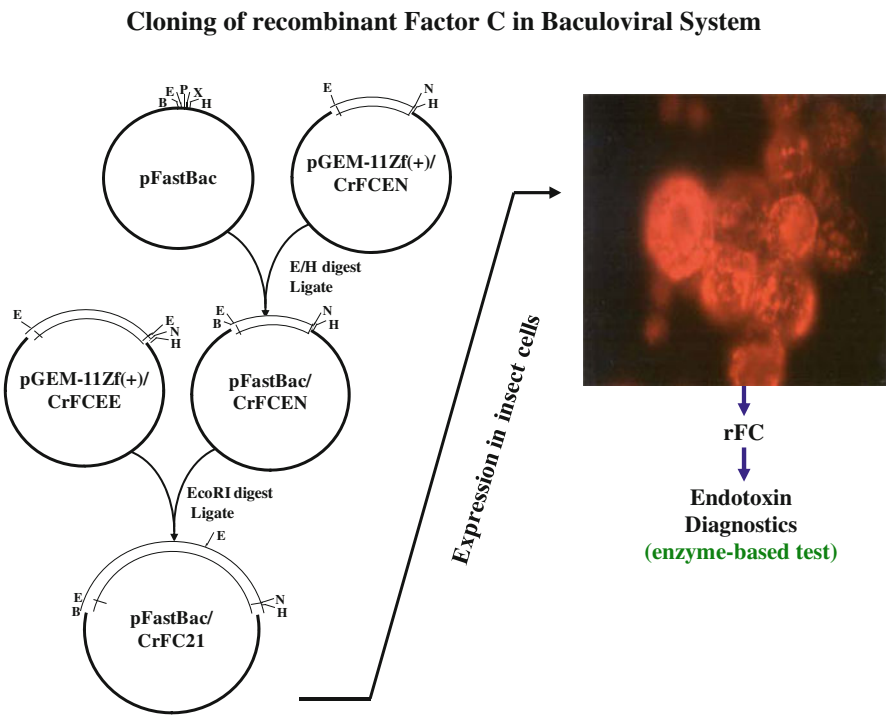

Fig. 9.5 Molecular cloning and expression of Factor C in the baculoviral system and expression in insect cells produced an rFC with remarkable sensitivity of 0.001 EU/mL endotoxin. (B: *BamHI,* E: *EcoRI*, X: *XhoI*, H: *HindIII,* P: *PstI*, N: *NcoI*)

endotoxin-binding site is located at the NH_2-terminal heavy chain region (Nakamura et al., 1988). The Japanese horseshoe crab (*Tachypleus tridentatus*) Factor C had been cloned in two separate and overlapping partial fragments (Muta et al., 1991). Ding et al. (1995) cloned the full length Factor C, originally in the *E. coli* and then recloned and expressed the recombinant Factor C (rFC) in different hosts, including yeast, *Saccharomyces cerevisiae* (Ding et al., 1997; Pui et al., 1997), *Pichia pastoris* and mammalian cell line, Cos-1 (Roopashree et al., 1996, 1997) and insect cells using the baculoviral system (Ding and Ho, 2001) (Fig. 9.5).

9.10 Development of a Quantitative Endotoxin Assay

Based on rFC, a modern and yet simple, rapid, specific and sensitive diagnostic test for endotoxin has been developed (Ding and Ho, 2001). rFC is a proenzyme until it encounters trace levels of endotoxin where it unequivocally exhibits full enzymatic activity, hence, acting as a very sensitive and specific biosensor for endotoxin. The resulting activated rFC acts as a catalyst to hydrolyse a synthetic substrate to form a quantifiable product, which measures the level of endotoxin. A fluorimetric assay

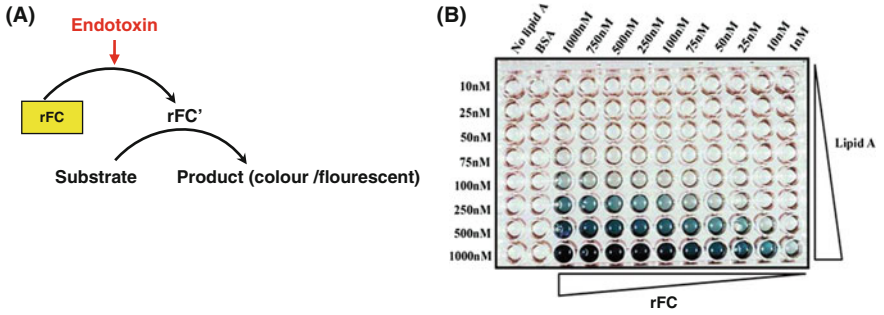

Fig. 9.6 Recombinant Factor C (rFC) is activated by endotoxin into rFC'. (**a**) A single-step activation of rFC which hydrolyses a substrate to yield either a colored (colorimetric assay) or fluorescent (fluorimetric assay) product, hence, (**b**) reporting on the presence and quantity of endotoxin present in samples (a colorimetric assay, in this case)

for endotoxin uses rFC zymogen, which, on activation by endotoxin, hydrolyses a fluorigenic 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 40 nm. Raising the amount of rFC in the assay was found to further increase the sensitivity of the detection of endotoxin from 0.005 EU/ml to 0.001 EU/ml (Ding and Ho, 2001). A comparison of rFC with commercial LAL, under the same assay conditions showed rFC to have lower background reading and a more sensitive response to endotoxin (Ding and Ho, 2001).

A colorimetric assay was also 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. A further demonstration that rFC is capable of detecting endotoxin (Ding and Ho – US patent filed no: 6,645,724B1) is depicted in Fig. 9.6. Compared to the coagulation cascade that occurs in the LAL, which is present in the conventional LAL-assay, the rFC affords a rapid and high-throughput assay in one single step. This reaction also obviates any potential interference of the specificity for LPS detection since no other blood proteins are present in the rFC.

9.11 Commercialization of the Endotoxin Detection Kit – The Route to PyroGene and Pyrosense

A microfluorimetric or microcolorimetric assay integrated into the rFC assay has allowed high-throughput screens of LPS in multiple samples. Currently, the rFC is used in an LPS-assay to sensitively and specifically detect LPS (Ding and Ho, 2001). The rFC has been incorporated into the PyroGene kit, which was launched in 2004 (Cambrex Inc., USA). Recently, Lonza Inc. (USA) applied rFC in PyroSense, which has potentials for continuous online monitoring of endotoxin in water and other fluids used for large-scale production of biomedical products.

The endotoxin test has a large market in drug companies that use LAL to detect endotoxin contamination in injectable products. For quality assurance and validation, every pharmaceutical company uses it for process monitoring. Furthermore, medical device firms require this test to ensure that catheters, pacemakers and other implantable devices are endotoxin-free. In gene therapy, where isolated plasmid DNA are used as therapeutics, international regulatory agencies are obliged to set stringent guidelines with regard to endotoxin levels (Levy et al., 2000). With this great impact on the future therapeutic and diagnostic approaches, and the growing need for the removal of endotoxins, the advent of rFC-based PyroGene test is timely to provide a sensitive, rapid and quantitative diagnostic for endotoxin. The rFC serves as a perpetual source of a genetically engineered biosensor for LPS. Finally, the horseshoe crab species should not need to be harvested for biomedical consumption. Towards an "environmentally-friendly science," the genetic engineering feat has contributed not only to the conservation of this "living fossil", but also to a more accurate, more sensitive and reliable, and less variable endotoxin test for the human healthcare industry.

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