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Biotechnology and Biomedicine - a Case Study with Recombinant Limulus Factor C

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Biotechnology "embraces everything from the production of recombinant proteins to the use of biological molecules as components of nanotechnology". Strategic-basic research in biotechnology is crucial to boosting world economy and creating jobs in the Life Sciences. Biotechnology will be the benefit to be drawn from biomedical life science research, which promises to be the new pillar of economy. Although the turnaround time for life science research products is painstakingly slow, the fruits of one such labour (Fig. 1), is being reaped for worldwide biomedical applications.

Bacterial Endotoxin - a Recalcitrant Biological *Enemy*

Lipopolysaccharide (LPS), a bacterial pyrogen, also known as endotoxin, is an integral component of Gram negative bacterial cell membrane. It is a ubiquitous and indomitable chemical health hazard which continues to challenge the biomedical industry. LPS directly stimulates host macrophages to secrete a wide array of inflammatory cytokines (Kreutz *et al.*, 1997). During Gram negative bacterial sepsis (Karima *et al.*, 1999), excessive release of these cytokines leads to multiple organ failure and death (Tracey & Lowry, 1990). Therefore, pharmaceutical products, parenteral fluids for human injection and medical devices / implants have to be critically tested and ensured to be free of LPS prior to use. Thus, reliable endotoxin diagnostics and therapeutics are urgently sought after.

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Limulus Amoebocyte Lysate (LAL) - the First Generation Diagnostic for Endotoxin

LPS-induced coagulation cascade (Fig. 2) of the Limulus amoebocyte lysate (LAL) represents an important defense mechanism used by horseshoe crabs against invasion of Gram-negative bacteria (Armstrong & Rickles, 1982). LAL has been widely used for decades as a tool for detecting LPS in pharmaceuticals and parenterals (Novitsky, 1994). Thus, since the mid 1970's, the Americans and Chinese have been producing LAL by procurring the life-saving blue blood from routinely harvested limulus. The limulus has thus been commercially exploited for worldwide biomedical needs. The widespread use of LAL has almost completely overtaken the use of rabbit pyrogen test, which was another Pharmacopoiea-approved method of testing for presence of LPS.

Genetic-engineering of Limulus Factor C - the Rationale

For more than 25 years, some major US biotechnology companies have been marketing the natural lysate from the limulus, to research institutes, hospitals, pharmaceutical companies. However, there are several setbacks to the use of LAL. Firstly, batch-to-batch and seasonal variations in LAL cause differential sensitivity to LPS. Secondly, lack of specificity for endotoxin is compounded by fungal contaminant, (1~3) β-D glucan, which switches on the alternate coagulation pathway (Iwanaga *et al.*, 1985), resulting in false positive test for pyrogen (Fig. 2). Thirdly, in the US Eastern Seaboard, the *Limulus polyphemus* eggs which are spawned during the summer season, and poorly hidden along the beach, provides a protein-rich banquet for migratory birds. This has led to tremendous decline in the

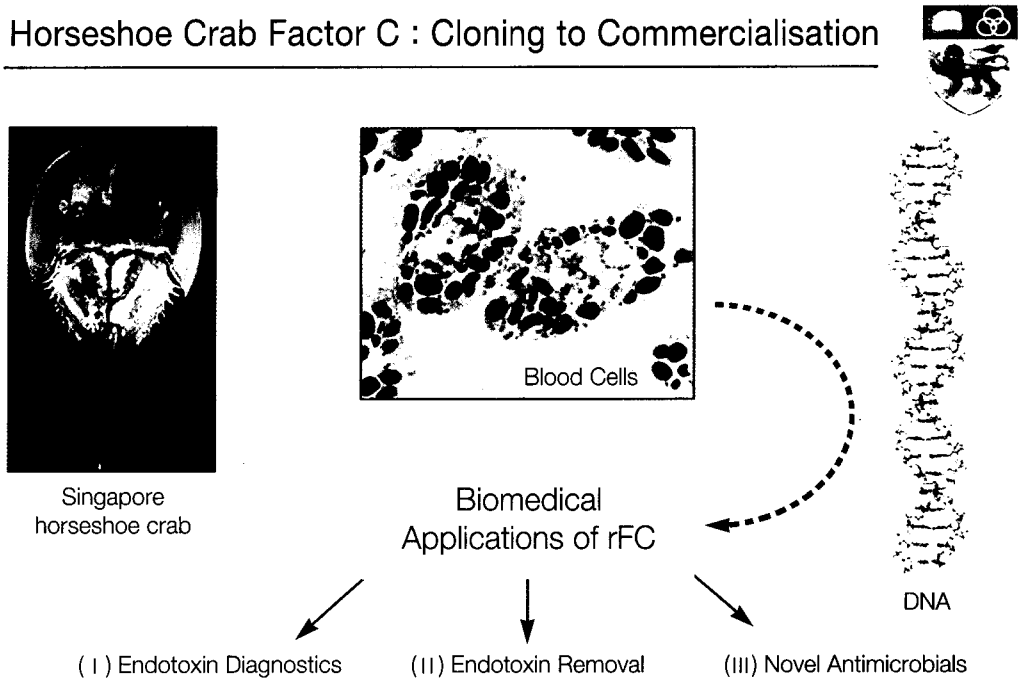


Fig. 1.

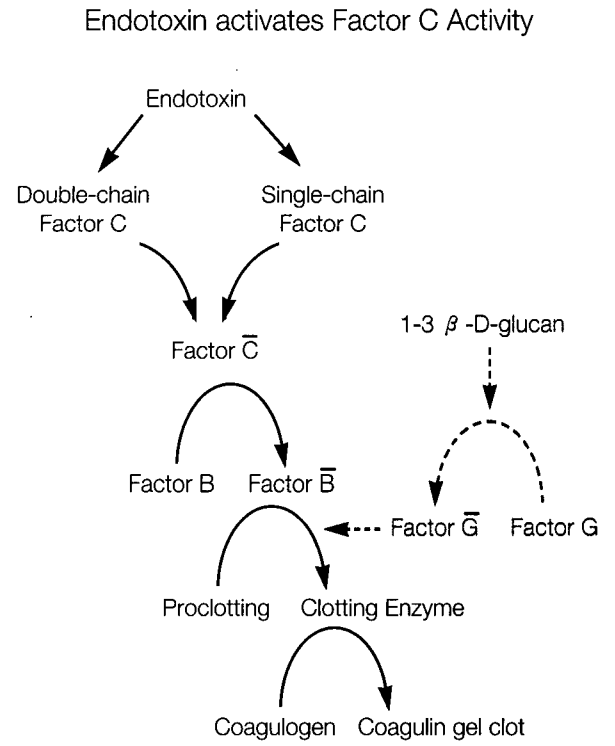
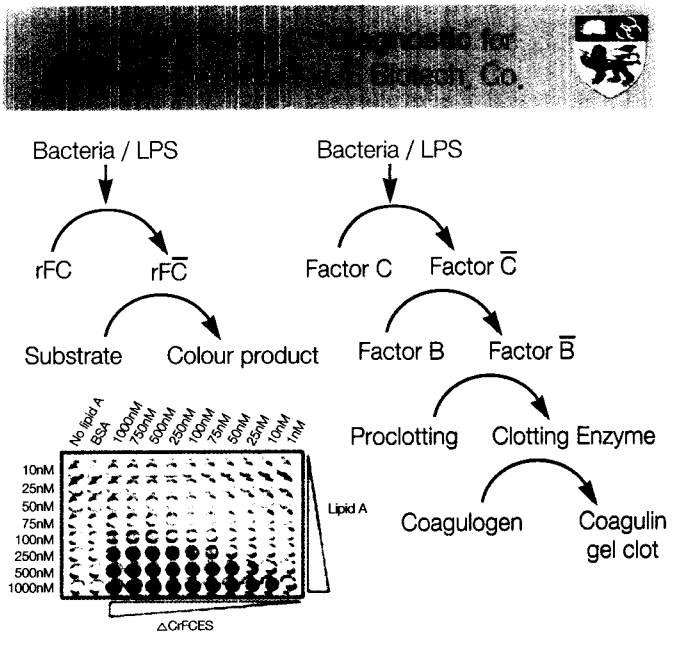


Fig. 2. Adapted from Ding *et al.*, (1993) Biochim. *et* Biophys Acta 1202: 149-156



Advantages of rFC over conventional LAL

- Cost Effective
- Rapid, Specific & Sensitive test
- High throughput screen
- Standardized supply
- Environmentally - friendly

Fig. 3.

horseshoe crab population (Widener & Barlow, 1999). To worsen this acute loss of potential new generations of limulus to the natural food web, the horseshoe crab has been over-harvested for commercial exploitation of LAL, which further endangers the species. In Japan, the *Tachypleus tridentatus* has been pronounced endangered (Sekiguchi & Nakamura, 1979). These near-extinction warnings and drawbacks have prompted us to look into long term alternatives like genetic engineering of lysate proteins such as Factor C, the first serine protease of the coagulation cascade which is enzymatically-activated by LPS (Ding *et al.*, 1993). Factor C functions as a biosensor that responds to femtogram levels of LPS (Ho, 1983). It is therefore conceivable that Factor C has an LPS-binding region that exhibits exceptionally high affinity for LPS. Consequently, recombinant Factor C (rFC) can be utilised to detect LPS. We have cloned (Ding *et al.*, 1995; US Patent No. 5,716,834) and expressed Factor C cDNA in various heterologous hosts (Roopashree *et al.*, 1995; Roopashree *et al.*, 1996; Ding *et al.*, 1997; Pui *et al.*, 1997; Roopashree *et al.*, 1997 a & b; Wang *et al.*, 2001).

Trials and Tribulations in Cloning and Expressing Biologically-functional rFC

The simplicity in the scientific rationale for cloning Factor C seems to offset the tremendous challenge of the molecular and biotechnological tasks. This work stems from a critical need to create a new generation of genetically-engineered Factor C enzyme for detection of picogramme levels of endotoxin. Being plagued by the ubiquity of endotoxin in laboratory materials, an initial major challenge was to establish pyrogen-free conditions for all experiments. Using basic tools of molecular and cellular biology and state-of-the-art and cutting-edge technologies, the Factor C cDNA was cloned. The first yeast clones produced high levels of rFC which is capable of binding endotoxin (Ding *et al.*, 1997; Pui *et al.*, 1997), but was not enzymatically activated by binding LPS. Neither did subsequent expression of rFC in mammalian cells yielded any functional recombinant protein (Roopashree *et al.*, 1997a). At such times, some basic knowledge in zoology certainly helps molecular biologist during such *sophisticated* failures. Since the horseshoe crab is an arthropod, and is more closely related to insects than crustaceans, our effort to re-clone the Factor C cDNA into insect cells, using the baculoviral system paid off with a significant breakthrough in expression of fully-functional rFC which is capable of specifically recognising endotoxin with remarkable sensitivity to LPS, at 0.001 EU/ml. Thus rFC represents a convenient enzymatic biosensor for trace levels (10^{-15} g) of endotoxins (Ding & Ho, 2001). There are many obvious advantages with the use of rFC over that of conventional LAL (Fig. 3). This clone has been out-licensed to a US Biotechnology company for scale-up culture, high level production and downstream processing of rFC for formulating a new generation of endotoxin diagnostic kit. Using rFC, a novel micro enzyme-based assay (US Patent Application No: 09/287,368) was developed for high throughput screens of pyrogen in pharmaceuticals and parenterals.

Conclusion: Save the "Living Fossil" - rFC is Environmentally-friendly

The horseshoe crab which has existed over the millennia, is dubbed a "living fossil". The importance of innovation, from basic molecular understanding of genes cloned from such a marine organism, to biotechnological applications cannot be overemphasized. The rFC clones are a robust and perpectual source of modern LAL that is readily prepared and will overcome all the drawbacks suffered by conventional LAL. This is the winning formula for both man and the horseshoe crab. There will be no need for harvesting the horseshoe crab since we can produce rFC in the lab test tubes. Thus, life science research in the area of molecular marine biotechnology provides a means to save the "living fossil" from extinction, and at the same time, we draw benefits for human healthcare.

Future Perspectives: Novel Biosensors & Therapeutics

Deletion constructs of rFC (SSCrFCES) encompassing the N-terminal 38 kDa region of Factor C, containing multiple LPS binding sites. This domain has been subcloned into a novel secretory system (Tan *et al.*, 2002) and expressed in drosophila cells in scaled-up cultures of >20 litres. Functional studies carried out on the SSCrFCES protein shows remarkable intricacies of binding properties with LPS, thus, explaining exceptional sensitivities of rFC for LPS (Tan *et al.*, 2000a). Further investigation of various deletion mutants containing individual LPS-binding motifs called sushi 1, 2 & 3, revealed stable monomeric proteins

with unsurpassed sensitivity and binding capacity for LPS (Tan *et al.*, 2000b). Short antimicrobial peptides designed based on the LPS-binding motif within the sushi domains were found to be efficacious in killing various bacterial pathogens (Yau *et al.*, 2001).

Since LPS is the major upstream initiator of inflammatory responses leading to septic shock, detailed understanding of the molecular interactions between LPS and target host proteins would be beneficial. Lipid A, the membrane anchor of LPS, is the biologically potent moiety of LPS (Kotani *et al.*, 1985). Thus, using computational simulations to rationalize the observed dependence of the LAL assay on the number and position of the phosphate groups in the lipid A moiety, Frecer *et al.* (2000a) have compared two simple mechanistic models of lipid A action on a membrane bound protein receptor on the host cell. By such studies, these workers proposed strategies for rational design of anti-endotoxin agents (Frecer *et al.*, 2000b).

Additionally, by rational design based on LPS-binding domain of Factor C, LPS-binding site (s) have been introduced into the EGFP molecule by site-directed mutagenesis. Thus, EGFPi mutants have been cloned. Functional EGFPi mutants have been expressed, containing high binding affinity for LPS. This EGFPi-LPS molecule provides a revolutionary fluorescent LPS-biosensor (Goh *et al.*, 2002), which may also have therapeutic applications.

REFERENCES

1) Armstrong PB and Rickles FR (1982): Endotoxin-induced degranulation of the Limulus amoebocyte. *Exp Cell Res*, **140:** 15-24.

2) Ding JL, Navas MAA and Ho B (1993): Two forms of Factor C from the amoebocytes of *Carcinoscorpius rotundicauda*: purification and characterisation. *Biochim et Biophys Acta*, **1202:** 149-156.

3) Ding JL, Navas MAA and Ho B (1995): Molecular cloning and sequence analysis of Factor C cDNA from the Singapore horseshoe crab, *Carcinoscorpius rotundicauda. Mol Marine Biol Biotech*, **4:** 90-103.

4) Ding JL, Chai C, Pui AWM and Ho B (1997): Expression of full length and deletion homologues of *Carcinoscorpius rotundicauda* Factor C in *Saccharomyces cerevisiae*: immunoreactivity and endotoxin binding. *J Endotoxin Res*, **4:** 33-43.

5) Ding JL and Ho B (2001): New era in pyrogen testing. *Trends in Biotechnol*, **19:** 277-281.

6) Ho B (1983): An improved Limulus gelation assay. *Microbios Letts*, **24:** 81-84.

7) Frecer V, Ho B and Ding JL (2000a): Molecular dynamics study on lipid A from *Escherichia coli*: insights into its mechanism of biological action. *Biochim et Biophysica Acta*, **1466:** 87-104.

8) Frecer V, Ho B and Ding JL (2000b): Interpretations of biological activity data of bacterial endotoxins by simple molecular models of mechanism of action. *Eur J Biochem*, **267:** 837-852.

9) Goh YY, Frecer V, Ho B and Ding JL (2002): Rational design of green fluorescent protein mutants as biosensor for bacterial endotoxin. Protein Engnr (in press).

10) Iwanaga S, Morita T, Miyata T and Nakamura T (1985): Haemolymph coagulation in Limulus. In: Microbiology, Eds L. Leive *et al.*, American Society of Microbiology, Washington, pp. 29-32.

11) Karima R, Matsumoto S, Higashi H and Matsushima K (1999): The molecular pathogenesis of endotoxic shock and organ failure. *Mol Med Today*, **5:** 123-132.

12) Kotani S, Takada H, Tsujimoto M, Ogawa T, Takahashi I, Ikeda T, Otsuka K, Simmauchi H, Kasai N, Mashimo J, Nagao S, Tanaka S, Harada K, Nagaki K, Kitamura H, Shiba T, Kusumoto S, Imoto M and Yoshimura H (1985): Synthetic lipid A with endotoxic and related biological activities comparable to those of a natural lipid A from an *Escherichia coli* RE-mutant. *Infect Immun*, **49:** 225-237.

13) Kreutz M, Ackermann U, Hauschildt S, Krause SW, Riedel D, Bessler W and Andreesan R (1997): A comparative analysis of cytokine production and tolerance induction by bacterial lipopeptides, lipopolysaccharides and *Staphylococcus aureus* in human monocytes. *Immunology*, **92:** 396-401.

14) Levin J, Tomasulo PA and Oser RS (1970): Detection of endotoxin in human blood and demonstration of an inhibitor. *J Lab Clin Med*, **75:** 903-911.

15) Novitsky TJ (1994): Limulus amoeboycte lysate (LAL) detection of endotoxin in human blood. *J Endotoxin Res*, **1:** 253-263.

16) Pui AWM, Ho B and Ding JL (1997): Yeast recombinant Factor C from horseshoe crab binds endotoxin and causes bacteriostasis. *J Endotoxin Res*, **4:** 391-400.

17) Roopashree SD, Chai C, Ho B and Ding JL (1995): Expression of *Carcinoscoripus rotundicauda* Factor C cDNA. *Biochem Mol Biol Intl*, **35:** 841-849.

18) Roopashree SD, Ho B and Ding JL (1996): Expression of *Carcinoscorpius rotundicauda* Factor C in *Pichia pastoris*. *Mol Marine Biol Biotech*, **5:** 334-343.

19) Roopashree SD, Ho B and Ding JL (1997a): Recombinant COS-1 cells express *Carcinoscorpius rotundicauda* Factor C. *Biotechnol Letts*, **19:** 357-361.

20) Roopashree SD, Ho B and Ding JL (1997b): The cys-rich and EGF-like domains of *C. rotundicauda* Factor C yields soluble fusion protein with GFP. *Biotechnology Letts* **19:** 1147-1500.

21) Sekiguchi K and Nakamura K (1979): Ecology of the extant horseshoe crabs. In: Biomedical Applications of the Horseshoe Crabs (*Limulidae*), Eds., Cohen *et al.*, Alan R Liss, New York, pp. 37-49.

22) Tan NS, Ho B and Ding JL (2000a): High affinity LPS-binding domain (s) in recombinant Factor C of a horseshoe crab neutralises LPS-induced lethality. *FASEB J*, **14:** 859-870.

23) Tan NS, Ng PML, Yau YH, Chong PK, Ho B and Ding JL (2000b): Definition of endotoxin-binding sites in horseshoe crab Factor C recombinant sushi proteins and neutralisation of endotoxin by sushi peptides. *FASEB J*, **14:** 1801-1813.

24) Tan NS, Ho B and Ding JL (2002): Engineering a novel secretion signal for cross-host recombinant protein expression. Protein Engnr (in press).

25) Tracey KJ and Lowry SF (1990): The role of cytokine mediators in septic shock. *Adv Surg*, **23:** 21-56.

26) Wang J, Ho B and Ding JL (2001): Functional expression of full length limulus Factor C in stably transformed Sf9 cells. *Biotechnology Letters*, **23:** 71-76.

27) Widener JW and Barlow RB (1999): Decline of a horseshoe crab population on Cape Cod. *Biol Bull*, **197:** 300-302.

28) Yau YH, Ho B, Tan NS and Ding JL (2001): The high therapeutic index of Factor C sushi peptides: potent antimicrobials against *Pseudomonas aeruginosa*. *Antimicrobial Agents Chemotherapy*, **45:** 2820-2825.