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Flow cytometric analysis of crayfish haemocytes activated by lipopolysaccharides

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Abstract

Lipopolysaccharides (LPS) from Gram-negative bacteria are strong stimulators of white river crayfish, *Procambarus zonangulus*, haemocytes in vitro. Following haemocyte treatment with LPS and with LPS from rough mutant R5 (LPS Rc) from *Salmonella minnesota*, flow cytometric analysis revealed a conspicuous and reproducible decrease in cell size as compared to control haemocytes. These LPS molecules also caused a reduction in haemocyte viability as assessed by flow cytometry with the fluorescent dyes calcein-AM and ethidium homodimer. The onset of cell size reduction was gradual and occurred prior to cell death. Haemocytes treated with LPS from *S. minnesota* without the Lipid A moiety (detoxified LPS) decreased in size without a reduction of viability. The action of LPS on crayfish haemocytes appeared to be related to the activation of the prophenoloxidase system because phenoloxidase (PO)-specific activity in the supernatants from control and detoxified LPS-treated cells was significantly lower than that from LPS and LPS-Rc treated cells ($P \leq 0.05$). Furthermore, addition of trypsin inhibitor to the LPS treatments caused noticeable delays in cell size and viability changes. These patterns of cellular activation by LPS formulations indicated that crayfish haemocytes react differently to the polysaccharide and lipid A moieties of LPS, where lipid A is cytotoxic and the polysaccharide portion is stimulatory. These effects concur with the general pattern of mammalian cell activation by LPS, thereby indicating common innate immune recognition mechanisms to bacterial antigens between cells from mammals and invertebrates. These definitive molecular approaches used to verify and identify mechanisms of invertebrate haemocyte responses to LPS could be applied with other glycoconjugates, soluble mediators, or xenobiotic compounds.

Keywords: Crayfish; Flow cytometry; Haemocyte; Innate immunity; Lipopolysaccharide

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1. Introduction

Lipopolysaccharides (LPS) are amphiphilic molecules localised in the outer leaflet of the outer membranes of Gram-negative bacteria. These molecules are highly antigenic and have endotoxic properties [1]. Antigenicity is associated with the polysaccharide portion of the molecule and the endotoxicity with the lipid moiety [1–3]. The endotoxicity of LPS is mediated by the secretion of inflammatory factors from immune reactive cells such as macrophages [2–5]. Interestingly, LPS recognition in mammals requires Toll-like receptor 4, an evolutionarily conserved surface protein. Signalling through this receptor requires adaptor proteins, kinases, and transcription factors that have counterparts in invertebrates such as *Drosophila* [6]. Although there are controversial views regarding the way mammalian cells recognize LPS [7], it is clear that cellular recognition of LPS generates a complex cascade of intracellular signals [8].

The innate recognition of LPS as nonself molecules is a common feature among crustaceans. Exposure to LPS causes exocytosis and activation of proclotting enzymes in *Limulus* haemocytes [9]. In vivo administration of LPS causes a decrease in the number of circulating haemocytes in several crustaceans [10]. Documented as being important in the recognition and defence system of arthropods [11], the prophenoloxidase activating system (proPO) can be converted to active phenoloxidase (PO) by LPS [12,13]. Activation of proPO from crayfish haemocyte lysates (HLS) requires lipid A acting in concert with some core polysaccharides of LPS because detoxified LPS (LPSdex), lipid A, or its sugar backbone β-D-GlcNAc-[1 → 6]-D-GlcNAc (compound X) acting alone failed to render proPO active [12]. This pattern of activation suggests that the polysaccharide portion of LPS is important in conditioning the endotoxic activity of lipid A of LPS in crayfish. The recognition of LPS in crustaceans might be mediated by an LPS and β-1, 3-glucan-binding protein (LGBP) identified in crayfish [14] and shrimp [15]. LGBP can bind LPS in vitro, but it is still not clear what role LPS plays in proPO activation.

Based on the observations of proPO activation by LPS in crayfish haemocyte lysates [12,13,16], a better delineation of the effect of LPS on the cellular activation of crayfish haemocytes is needed. Flow cytometry was used to assess the patterns of cell activation. This technique provides an objective, reproducible, and sensitive characterization of a large number of haemocytes in a short time with the added capability of multiparametric analysis of individual cells in real time [17,18]. The results of this study showed that LPS and LPS derivatives induce different responses by crayfish haemocytes and these responses appear to be mediated by secreted or extracellular proPO.

2. Materials and methods

2.1. Chemicals and reagents

Lipopolysaccharide from *Salmonella minnesota*, LPS from *S. minnesota* R5 Rc mutant (LPS Rc), LPS detoxified (LPSdex) from *S. minnesota*, trypsin (bovine pancreas), trypsin inhibitor (soybean type I-S), 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES), L-3,4-dihydroxyphenylalanine (L-DOPA), and trypan blue (0.4%) were obtained from Sigma Chemical Co. (St. Louis, MO). The sheath fluid (FACSFlow) was obtained from Becton Dickinson Immunocytometry Systems Co. (BDIS) (San Jose, CA). Calcein-AM and ethidium homodimer (EthD-1) dyes were from Molecular Probes, Inc. (Eugene, OR), obtained as a LIVE/DEAD® viability/cytotoxicity kit. Protein concentrations were determined by using a Bio-Rad Protein Assay System (Bio-Rad, Richmond, CA) adapted for a 96-well-microtitre plate assay with bovine serum albumin as the standard.

2.2. Preparation of haemocytes

Procambarus zonangulus were generously provided by Dr J. Huner from the Crawfish Research Center, University of Louisiana at Lafayette, Lafayette, LA. Animals were maintained in the laboratory at the U.S. Geological Survey, National Wetlands Research Center, Lafayette, LA in a freshwater recirculation system at 24 °C, and acclimated at least 2 weeks prior to use. Only apparently healthy male animals weighing 30–55 g were used.

Haemolymph was extracted from each crayfish after anaesthetising for 15 min on ice. Animals were bled by using a 3- or 5-ml syringe with a 26-gauge needle (Becton Dickinson, Franklin Lakes, NJ) containing ice-cold anticoagulant buffer (AB), pH 4.6 [19] to yield a 1:2 ratio of AB:haemolymph. Diluted haemolymph was distributed into two prechilled 1.5 ml sterile microcentrifuge tubes and centrifuged at $735 \times g$ for 5 min at 4 °C. The supernatant was discarded and the cell pellets were resuspended in ice-cold AB. Resuspended cells were pooled and centrifuged as before. The supernatant was discarded and the cell pellet resuspended in 1 ml of crayfish saline (CFS) [20] with 5 mM of HEPES (CFS-HEPES), pH 6.8. The osmolality (Wescor, Logan, UT) of AB and CFS-HEPES was maintained at approximately 400 mmol kg^{-1}. Cell counts and viability were determined by using light microscopy with a haemocytometer and trypan blue exclusion. Cell suspensions at 10^7 ml^{-1} with viabilities greater than 90% were then utilised in this study.

2.3. Activation of haemocytes

To assess their reactivity, isolated haemocytes in CFS-HEPES were incubated with LPS, LPSdex, and LPS Rc. All LPS and LPS derivatives were prepared as stocks of 10 mg ml^{-1} in saline (396 mmol kg^{-1}) and final experimental concentrations were 1 and 10 μg ml^{-1}. Reaction mixtures of haemocytes ($\sim 2 \times 10^6$ cells ml^{-1} final concentration) and the LPS treatments or saline controls were incubated at 24 °C in 1.5 or 2.0 ml siliconised microcentrifuge tubes (Sigma). At 10 or 20 min intervals during the incubation, aliquots were removed to 200 μl of CFS-HEPES in 12×75 mm disposable, round-bottomed culture tubes. These subsamples were immediately analysed by flow cytometry. Experiments were performed in triplicate on isolated haemocytes from individual animals. To investigate the involvement of serine protease action on the effects induced by LPS, trypsin inhibitor (TI) at a final concentration of 100 μg ml^{-1} was included in the 1 μg ml^{-1} LPS reaction mixture.

2.4. Flow cytometry

Flow cytometry was performed with a FACScan (BDIS) equipped with a single argon ion laser with filtered emission at 488 nm. Photomultiplier bandpass filters for fluorescence were 530 nm (green fluorescence) and > 650 nm (red fluorescence). The instrument was calibrated by using AutoComp software and CaliBRITE beads (BDIS). Forward (size) and side light scatter (granularity) data were collected on linear scales, and fluorescence data were collected on log scale. The voltage settings were FSC E00, SSC 400, FL1 548 log, and FL3 568 log. Data were ungated, and 10 000 events in total were acquired per subsample. Lysis II (BDIS) and WinList software (Verity Software House, Topsham, ME) were used to create logical regions for data analyses, and WinList was used for colour gating analyses of fluorescence data.

2.5. Cell viability

To assess cell viability, a two-colour fluorescent labelling protocol (Molecular Probes) was optimised for use. The membrane-permeable dye calcein-AM was prepared with the membrane-impermeable dye EthD-1 as a working stock solution of 100 μM each in CFS-HEPES. Aliquots of 1 μl from this stock solution were added to 100 μl subsamples of the cell reaction mixtures, resulting in a final concentration of 1 μM for both dyes. Reaction mixtures were incubated with dyes at 24 °C for 10 min and mixed with 200 μl of CFS-HEPES in 12×75 mm disposable, round-bottomed culture tubes prior to flow cytometry. Calcein-AM (diagnostic of live cells) is non-fluorescent until intracellular esterases hydrolyse the acetoxymethyl ester groups and trap the dye inside the cells producing a green fluorescence (∼530 nm). Once inside the cell, EthD-1 (diagnostic of dead cells or cells with damaged membranes) binds nucleic acids producing a bright red fluorescence (>600 nm).

2.6. Phenoloxidase-specific activity

After the subsampling for flow cytometry, remaining cells in each of the reaction mixtures were immediately centrifuged at 735×*g* for 10 min at 4 °C. These treatment supernatants (TS) were transferred to siliconised microcentrifuge tubes and kept on ice. Phenoloxidase (PO)-specific activity was determined as described before [12], by using a volume of 50 μl from each TS. To render proPO active, trypsin (1 μl of a 10 mg ml^{-1} solution) was included in duplicate sets of TS. The chromogenic substrate was L-DOPA.

2.7. Statistics

Valid differences in cell size and viability between treatments with time were analysed by a General Linear Means procedure (PROC GLM) [21]. In order to test the source of difference within each parameter, a Duncan's Multiple Range test was applied. In order to compare rates of change, Student's *t*-test was used. Differences were considered significant at $P \leq 0.05$. Mean comparisons of PO-specific activity of TS were analysed by Student's *t*-test with significance at $P \leq 0.05$, and differences in activity were determined with analysis of variance at $P \leq 0.05$. Values represented the mean of at least three individual experiments, and standard error bars are shown in the figures.

3. Results

To evaluate the effects of LPS and LPS-derivatives on crayfish haemocytes, the light scattering properties of the cells were studied by flow cytometry. Cell size is measured by forward light scatter and side light scattering reflects granularity or cellular complexity [22]. These experiments employed cells from individual animals rather than pooled haemolymph from several animals, so changes in these properties [23] were not attributed to nonself recognition.

Preliminary flow cytometric data showed that a main effect of LPS on crayfish haemocytes was a change in cell size. Cell size change was traced by setting electronic gates on two regions: region of large cells (R1) and region of small cells (R2) in one parameter histograms of the size parameter (Fig. 1a). Viability was measured by two-parameter (FL1 and FL3) dot-plots in log scale, also divided into two regions: region of live cells (R1), and region of dead cells (R2) (Fig. 1b).

Throughout this experiment, the percentage of large cells in the control treatment remained fairly constant, with a mean of 80.6% in R1 and 19.3% in R2 (Fig. 2a). In the LPS treatment at 1 μg ml^{-1}, the percentage of large cells was 80.5% at time 0 min and decreased to 62.4% at 120 min, the last time point with enough cells to process (Fig. 2b). The change in cell size was similar with a 10-fold increase in LPS, whereby R1 was 81% at time 0 min, and decreased to 64.8% at 100 min, the last time point measured (Fig. 2c). For each of the LPS treatments, a reciprocal increase in the number of cells in R2 was seen (Fig. 2b,c). Duncan's Multiple Range test indicated significant differences in the sized populations between

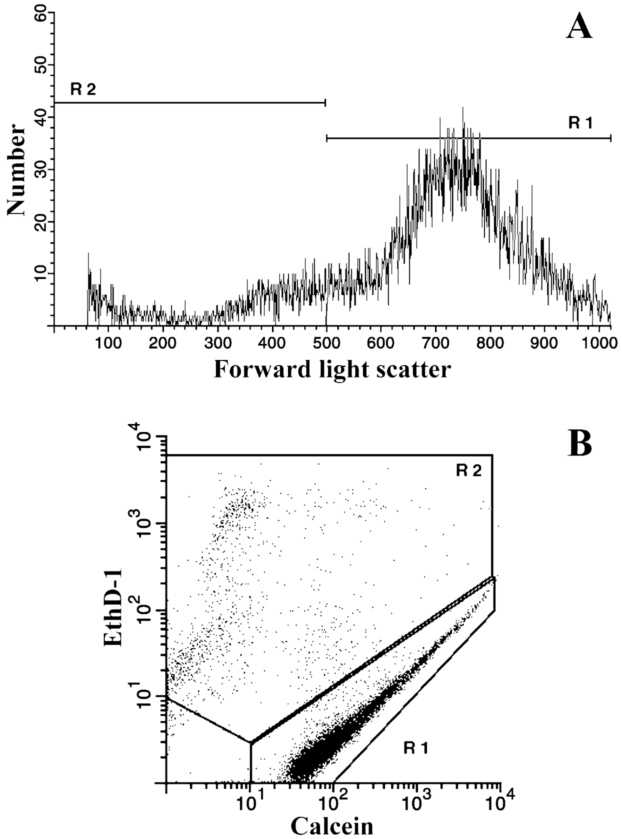

Fig. 1. (A) Typical one-parameter histogram illustrating forward light scatter (size) and relative cell number analysed per sample. Region 1 (R1) or large cells, and Region 2 (R2) or small cells, were arbitrarily demarcated. Histograms from each experiment were analysed by using the designated regions. (B) Two-parameter flow cytometric dot-plots of calcein-reacted live cell fluorescence (R1) versus ethidium-generated dead cell fluorescence (R2) used for assessing haemocyte viability.

the control and both LPS treatments (1 and 10 μg ml^{-1}), but no difference between the LPS treatments ($P \leq 0.05$).

A parallel time course analysis of cell viabilities from the same treatments showed that with the decrease in size of R1, there was a corresponding, yet delayed, decrease in viability (Fig. 2e,f). In the control treatment, the percentage of cells in the live (R1) and dead (R2) regions were 94.6% and 4.5%, respectively, throughout the experiment (Fig. 2d). When the cells were exposed to 1 μg ml^{-1} LPS, the percentage of cells at time 0 min in R1 and R2 were 93% and 5.6%, respectively (Fig. 2e). At 120 min, viable cells decreased to 54% and nonviable cells increased to 45%, respectively. A 10-fold increase in the LPS concentration showed similar shifts, where 94.4% and 4.7% of the cells were distributed between R1 and R2 at time 0 min, respectively (Fig. 2f). After 100 min of incubation, the cell percentages in these regions changed to 63% and 34.5%. As with cell size, significant differences in viabilities were noted between control and both LPS treatments (1 and 10 μg ml^{-1}), but no difference was seen between the LPS treatments ($P \leq 0.05$).

As structural differences in LPS molecules have been shown to effect the activation of proPO from HLS [12], haemocytes were incubated with the LPS derivatives LPS Rc (1 μg ml^{-1}) and LPSdex (10 μg ml^{-1}) (Fig. 3). Cell size and viability changes were determined as before. Duncan's test indicated that the large

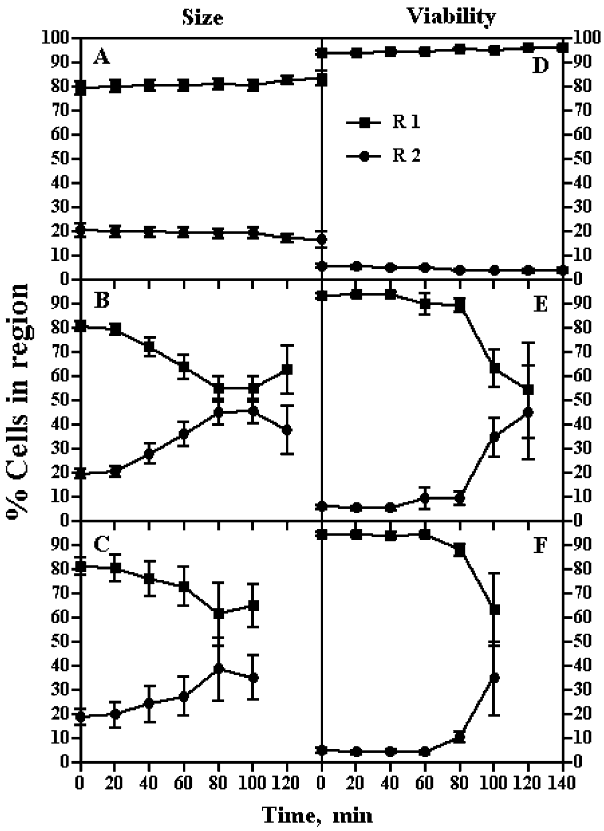

Fig. 2. Time course of size (a–c) and viability (d–f) changes of crayfish haemocytes activated by LPS from *Salmonella minnesota*. Crayfish haemocytes were incubated with LPS or crayfish saline control treatments and subsampled every 20 min for analysis by flow cytometry. Control cells (a,d); cells incubated with 1 μg ml $^{-1}$ (b,e) or with 10 μg ml $^{-1}$ (c,f) LPS from *S. minnesota*. For each time point 10 000 cells were analysed. At least three experiments were performed for each treatment. Error bars represent the standard error.

and small subpopulations in the CFS control treatment (Fig. 2a) were significantly different from the two LPS derivative treatments (LPS Rc and LPSdex), but not between the two derivatives ($P \leq 0.05$) (Fig. 3a,b). No significant difference in viability was noted between the control and LPSdex treatments (Figs. 2d and 3d), yet these two treatments were significantly different from LPS Rc ($P \leq 0.05$) (Fig. 3c).

To further investigate the involvement of a proteolytic cascade important in proPO activation, trypsin inhibitor (TI) was included in the 1 μg ml $^{-1}$ of LPS treatment (Fig. 4). Time course analysis of cell size and viability changes in the control treatment of TI showed a fairly constant mean percentage of cells throughout the experiment (Fig. 4a,d), being significantly different from those of the LPS and LPS plus TI treatment ($P \leq 0.05$) (Fig. 4b,c,e,f). The LPS plus TI treatment decreased the rate of changes in size and viability, allowing for additional time point measurements (Fig. 4c,f), although not significantly as compared with the LPS treatment (Fig. 4b,e), with $P = 0.07922$ for size, and $P = 0.06287$ for viability.

The PO specific activity was assayed in the TS remaining after the completion of flow cytometric analysis of controls and LPS treatments. The mean TS PO specific activity from control and LPSdex treatments was significantly lower than the mean values from the LPS and LPS Rc treatments ($P \leq 0.05$) (Table 1). The addition of trypsin further activated the proPO in all treatments so that mean specific activity values were not different among all treatments (Table 1) ($P \leq 0.05$).

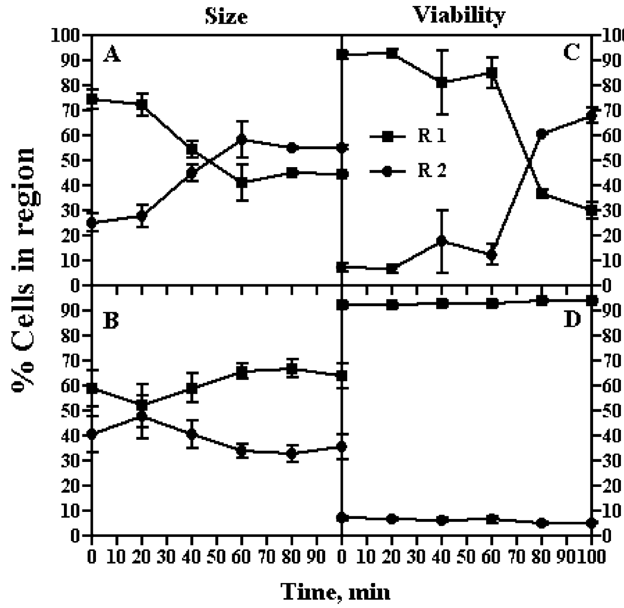

Fig. 3. Time course of size and viability changes of crayfish haemocytes incubated with 1 μg ml $^{-1}$ LPS Rc (a,c) and 10 μg ml $^{-1}$ LPSdex (b,d) from *S. minnesota*. Crayfish haemocytes were incubated with treatments and subsampled every 20 min for analysis by flow cytometry. For each time point 10 000 cells were analysed. At least three experiments were performed for each treatment. Error bars represent the standard error.

4. Discussion

Flow cytometry has been used to accurately ascertain differences and similarities in haemocyte subpopulations between bivalve [24] and prawn species [25], in detecting molt-related changes in haemocyte types in *Penaeus japonicus* [26], and in counting prawn *Penaeus* haemocyte types [27]. In this study, in order to objectively track individual crayfish haemocyte responses to specific microbial molecules, flow cytometric measurements were taken on cell size and viability with time of exposure. Crayfish haemocytes showed a strong response to LPS where the reduction in cell size correlated with cell death. Similar effects were observed when LPS from a mutant bacterial strain (Rc) was used. The Rc lipopolysaccharides are devoid of the O-antigen and have an incomplete core structure formed mainly by glucose and heptose linked to lipid A via 2-keto-3-deoxy-D-*manno*-octonate [28].

The similar action of LPS and LPS Rc against crayfish haemocytes suggests that the O-antigen polysaccharides are not responsible for cell death. This interpretation was supported by the opposite effect of LPSdex, where haemocytes did not lose viability relative to untreated control cells. Although there were no statistical differences between control and LPSdex viability assays, there were statistical differences between the sized populations, thereby reflective of the observed size changes in the LPSdex treatment without the concomitant loss of cell viability as with the other LPS treatments (see Figs. 2. and 3). The biochemical pathways leading to cell size reductions induced by LPSdex and LPS Rc are not yet defined. Taken together, these results imply that crayfish haemocytes are able to react differentially to the polysaccharide and lipid A moieties of LPS, where lipid A is cytotoxic and the polysaccharide portion stimulatory. This result agrees with the recognized endotoxic properties of lipid A in LPS [29,30], and the observed reduction of toxicity of deacylated LPS that still possesses potent inflammatory properties in humans [31,32].

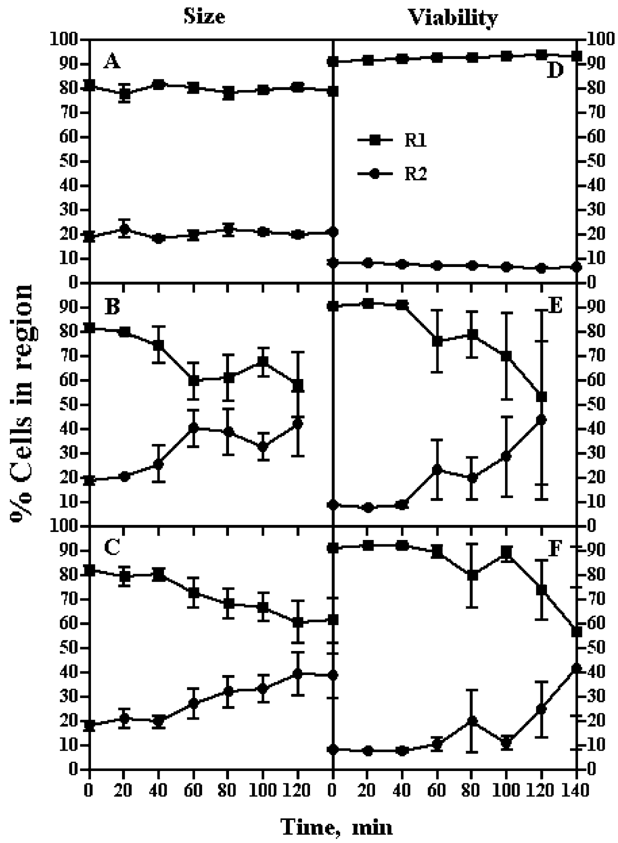

Fig. 4. Time course of size and viability changes of crayfish haemocytes incubated with 100 μg ml^{-1} trypsin inhibitor (TI) (a,d); 1 μg ml^{-1} LPS (b,e); and 1 μg ml^{-1} LPS plus 100 μg ml^{-1} TI (c,f). Crayfish haemocytes were incubated with treatments and subsampled every 20 min for analysis by flow cytometry. For each time point 10 000 cells were analysed. At least three experiments were performed for each treatment. Error bars represent the standard error.

The reaction of live haemocytes to LPS Rc, and LPSdex is similar to the reported effects of these molecules on the proPO activation from cell lysates, where LPS Rc was effective in activating the system and LPSdex was not [12]. This activation might be mediated by an endogenous haemocyte proteinase [13]. Since a serine proteinase is at the center of the cellular recognition cascade triggered by microbial molecules in invertebrates [33], trypsin inhibitor (TI) was included in the reaction mixtures. Although not statistically significant, this TI addition to the LPS treatment reproducibly decreased the rate of changes in size and viability, thereby resulting in additional measurable time points not attained with the LPS (1 or 10 μg ml^{-1}) or LPS Rc treatments. However, TI did not completely block cell size change and cell death, as seen when zymosan A was used as a stimulator [17]. Since PO-specific activity in the TS corresponds to the effects of LPS treatments on crayfish haemocytes (see below) and the activation of proPO by LPS is known to be mediated by a protease zymogen in haemocyte lysates [13], the putative serine protease cascade resulting in proPO activation by LPS may have been triggered inside haemocytes. As opposed to these experimental conditions in which live haemocytes were used, TI exposed to haemocyte lysates can halt proPO activation by microbial compounds [12], probably by direct interaction with the activator protease. Exocytosed ProPO is rendered active by LPS or other microbial molecules [16], but the mechanism of signalling by LPS,

Table 1
Phenoloxidase specific activity from supernatants of *Procambarus zonangulus* haemocytes collected after the final time point measured in flow cytometry experiments

Supernatant	PO specific activity (Δ492 per min mg^{-1} protein)	*n*
Control	0.51 ± 0.86^{1}	6
Control + trypsin	8.66 ± 0.86^{3}	6
LPS	1.68 ± 0.86^{2}	6
LPS + trypsin	8.01 ± 0.86^{3}	6
LPSdex	0.41 ± 0.94^{1}	5
LPSdex + trypsin	11.15 ± 0.94^{3}	5
LPS Rc	2.68 ± 1.22^{2}	3
LPS Rc + trypsin	7.17 ± 1.22^{3}	3

Values are mean ± SE for *n* number of experiments. Dissimilar superscripts indicate significant differences within the means ($P \leq 0.05$).

which leads to proPO secretion, is largely unknown. Activated proPO would lead to haemocyte degranulation and lysis [19,34], as with LPS and LPS Rc treatments.

To investigate whether PO was involved in the haemocyte response to LPS, its specific activity was measured in the TS at the end of each experiment. The PO specific activity was significantly higher from LPS-treated haemocytes (LPS and LPS Rc), in which cell size reduction and cell death occurred. PO specific activity in the LPSdex treatment was not different from control values, which corresponds to the lack of cell death in both of these treatments. These observations imply that the proPO system has a central role in the haemocytic reactions to LPS reported herein. A low level of activation was sufficient to trigger cellular responses, as mean specific activity values were rather low compared with those from another report [12]. Furthermore, addition of trypsin to all TS showed that the system was largely inactive. Similarly high specific activity values of trypsin-activated proPO in TS from control cells, as well as from treatments with LPS, LPS Rc, and LPSdex suggest that control haemocytes secreted proPO without any apparent microbial stimulus. Additionally, it follows that the LPS treatments did not remarkably increase further proPO secretion. Under the present experimental conditions, a microbial-independent proPO exocytosis pathway was apparent, and haemocytes reacted to LPS in a predictable pattern, as with mammals.

It is important to note that in all LPS and LPS Rc treatments, cell size reduction was gradual, but cell death occurred rapidly and at the final time points measured (see Figs. 2–4). These patterns suggest that crayfish haemocytes react to microbial challenges by a kind of innate recognition mechanism and that LPS is not simply cytotoxic. Lipopolysaccharides are known to trigger the exocytosis of the nonself recognition molecule p47 through a protein tyrosine phosphorylation pathway in insect haemocytes [35]. Lipopolysaccharide-dependent exocytosis of a proPO serine proteinase activator is also mediated by protein tyrosine phosphorylation [36]. The present study provides the basis for further characterisation of the molecular components of TS (as well as intracellular components) and possible associations with differential in vitro reactions by haemocytes to microbial compounds.

The present study opens the door for definitive molecular approaches to verify and identify the mechanisms of invertebrate haemocyte responses to LPS and other glycoconjugates, or to influences by soluble mediators and even xenobiotic compounds. Flow cytometry was used in this study as a reliable tool to study real-time functional cellular responses by invertebrate haemocytes to microbial insults. As only necrotic and late apoptotic cells take up membrane-impermeable DNA stains, future flow cytometric studies could be designed to distinguish the mode of cell death. In this study, preservation of membrane integrity and cell size reduction, rather than early cell rupture as seen in necrosis, are characteristic of early apoptotic events [37]. Subcutaneous inoculation of LPS has been shown to activate pro-apoptotic genes in mouse fibroblasts together with a significant increase of caspases 8 and 3 activation [38]. The processes described herein engender an improved understanding of defence responses in crustaceans, so they may be used to establish reliable indicators of health status and condition.

Innate immune recognition mechanisms are necessarily timely and efficient. Proinflammatory cytokines such as interleukin-1, interleukin-6, and tumour necrosis factor found in mammals are also suggested to be present in several invertebrates [39,40]. Comparative studies of innate immune responses to common bacterial antigens are valuable in understanding shared mechanisms of cell recognition within and between taxa, as such endeavours are more complicated with higher vertebrates because of the superimposed action of the adaptive immune system. Exploitation of such comparative studies with LPS has resulted in the discovery of mammalian homologues of *Drosophila* Toll proteins, important in the cell-signalling responses to microbial molecules [41].

Finally, these results have important commercial implications because β-glucans are proposed as immunostimulants in shrimp [42,43] and prawn [44] production. Previous in vitro experiments with zymozan A have shown it to be cytotoxic to haemocytes [17]. Compounds similar to LPSdex, that exert strong cellular activation without cellular toxicity, might then be proposed as strong candidates as immunostimulants for commercial aquaculture practices.

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