Short communication

Effect of crude lipopolysaccharide from *Escherichia coli* O127:B8 on the amebocyte-producing organ of *Biomphalaria glabrata* (Mollusca)

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**A B S T R A C T**

Lipopolysaccharide (LPS) is a pathogen associated molecular pattern (PAMP) to which the internal defense system (IDS) of both vertebrates and invertebrates responds. We measured the mitotic response of the hematopoietic tissue of the schistosome-transmitting snail, *Biomphalaria glabrata*, to crude LPS from *Escherichia coli* O127:B8. In a dose-response study, snails were injected with a range of concentrations of crude LPS, and mitotic figures were enumerated in histological sections of amebocyte-producing organ (APO) fixed at 24 h post-injection (PI) following a 6 h treatment with 0.1% colchicine. In APOs from Salvador strain snails, which are genetically resistant to infection with *Schistosoma mansoni*, LPS concentrations of 0.01 mg/ml and above triggered a large increase in mitotic activity, whereas in APOs from schistosome-susceptible NIH albino snails, concentrations of 0.1 mg/ml elicited a much smaller, but statistically significant increase. A time course study, without colchicine treatment, revealed that in Salvador APOs the mitotic response to 0.1 mg/ml occurred by 18 h PI, peaked at 24 h, and returned to control levels by 72 h; NIH albino APOs showed no detectible response. When Salvador APOs were exposed to crude LPS in vitro, no increase in mitotic activity occurred, a result suggesting the possible requirement for a peripheral tissue or hemolymph factor. The increased cell proliferation induced by crude LPS represents a novel systemic response of an invertebrate IDS to one or more PAMPs from a Gram-negative bacterium.

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1. Introduction

A major component of innate immunity is recognition of pathogen-associated molecular patterns (PAMPs) with pattern recognition receptors (PRRs) encoded in the germline (Janeway and Medzhitov, 2002). In mammals, lipopolysaccharide (LPS) from the Gram-negative bacterial cell wall is one of the most thoroughly investigated PAMPs, largely because of its role in septic shock (Cohen, 2002). LPS is recognized in association with several accessory proteins by a PRR, Toll-like receptor 4 (TLR4), which then initiates a signaling pathway leading to activation of the transcription factors NF-κB and LPS-induced TNF-α factor (LITAF), in turn resulting in proinflammatory cytokine secretion by macrophages and proliferation of B cells (Anderson et al., 1972; Cohen, 2002; Imler and Hoffmann, 2001; Janeway and Medzhitov, 2002; Nau et al., 2002; Tang et al., 2006).

Whether the innate internal defense system (IDS) of invertebrates mounts such a systemic response to LPS is not clear. The most intensively studied invertebrate IDS, that of *Drosophila melanogaster*, responds to Gram-negative bacterial challenge via an immune deficiency (IMD)-mediated signaling pathway, leading to activation of an NF-κB precursor, Relish, and secretion of antimicrobial peptides from the fat body (Lemaître and Hoffmann, 2007). However, this response is triggered not by LPS but instead by diaminopimelic acid-type peptidoglycan (PGN), which is a contaminant of some commercial source, crude (phenol-extracted, without further purification) LPS preparations (Kaneko et al., 2004).

In molluscs, hemocytes respond to an in vitro challenge with crude LPS, as shown by altered cell morphology (reviewed by Cohen and Kinney, 2007), superoxide anion production (Gorbushin and lakovleva, 2007) catecholamine release (Cao et al., 2004a,b), increased NO and IL-2 production (Li et al., 2010) and differential gene expression (Xu and Faisal, 2009). Exposure of whole organisms to Gram-negative bacteria or LPS leads to expression of transcripts for several potential antibacterial proteins, e.g., bactericidal/permeability-increasing protein, peptidoglycan recognition proteins, and Gram-negative bacteria binding protein (Adema et al., 2010; Gonzalez et al., 2007; Zhang et al., 2009).

**Abbreviations:** ANOVA, analysis of variance; APO, amebocyte-producing organ; CBS, Chernin's balanced salt solution; IDS, internal defense system; IMD, immune deficiency; LITAF, LPS-induced TNF-α factor; LPS, lipopolysaccharide; PAMP, pathogen-associated molecular pattern; PGN, peptidoglycan; PI, post-injection; PRR, pattern recognition receptor; TLR, Toll-like receptor; TNF, tumor necrosis factor.

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Whether LPS alone is responsible for the above effects is unclear. Relative to possible signaling mechanisms, evidence for Toll, IMD and LITAF pathways has been reported in several species of molluscs (De Zoysa et al., 2010; Wang et al., 2011; Zhang et al., 2007).

In the schistosome-transmitting snail Biomphalaria glabrata, the amebocyte-producing organ (APO), a collection of follicles of hemocytes progenitors located in a sinus of the anterior wall of the pericardial sac (Jeong et al., 1983), shows increased mitotic activity in response to infection with larval trematodes (Lie et al., 1975) or injection with trematode excretory-secretory products or freeze-thaw extracts (Noda, 1992; Sullivan et al., 2004). The response to parasite freeze-thaw extracts also occurs in vitro, and may involve protein kinase-C-mediated intracellular signaling pathways (Salamat and Sullivan, 2008, 2009). We report here that the APO of B. glabrata responds in vivo to crude LPS from Escherichia coli.

2. Materials and methods

2.1. Snails

The schistosome-susceptible NIH albino and the schistosome-resistant Salvador strains of B. glabrata were raised in aerated aquaria and fed Romaine lettuce ad libitum, as described previously (Sullivan et al., 2004). Snails measured approximately 9.5–10.5 mm in shell diameter.

2.2. LPS

Crude LPS from E. coli O127:B8 (Sigma, St. Louis, MO) was dissolved in Chernin’s balanced salt solution (Chernin, 1963) (CBSS) and stored at −20 °C. According to the vendor, this material is isolated by phenol extraction of ATCC strain 127240, and in addition to LPS it contains both RNA and denatured protein; peptidoglycan content is not specified. Hereafter, we refer to this crude LPS preparation simply as LPS.

2.3. LPS injection

To test for a dose-dependent response, NIH albino and Salvador snails were injected with 5 μl of CBSS (controls) or CBSS containing 0.001, 0.01, 0.1, 1, or 10 mg/ml LPS. Assuming a total liquid volume in 10 mm snails of 60 μl (Sullivan and Cheng, 1976) and no leakage of the injection fluid, calculated whole body concentrations of LPS were estimated at 76.9 ng/ml at the lowest dose and 769 μg/ml at the highest. The injection apparatus consisted of a glass capillary tube that was pulled over a flame to a fine tip, mounted in a micromanipulator, and attached to a syringe with plastic tubing. Injections were made through a hole in the shell overlying a hemolymph sinus on the left side, anterior to the digestive gland as described previously (Sullivan et al., 2004) with the following modification. The tip of the capillary tube was advanced until it just made contact with, and slightly depressed the body wall. Rubber cement was then applied, and after the cement began to solidify (approximately 1 min), the tube was then advanced into the hemocoeal. This minor change greatly reduced the problem of bleeding encountered in the previous study, in which rubber cement was applied subsequent to penetration of the body wall (Sullivan et al., 2004). Snails were then incubated in individual 500 ml containers at 27 ± 2 °C for 18 h with lettuce, followed by incubation in 4 ml of 0.1% colchicine in individual 35 mm plastic Petri dishes for 6 h without food. At 24 h post-injection (PI) the pericardial sac, containing the APO, was dissected from each snail and fixed in Bouin’s fluid. Colchicine was used in an effort to enhance sensitivity of the assay by increasing the number of mitotic figures observed (Sullivan, 2007). At each concentration, pericardial sacs from 10 NIH albino or 20 Salvador snails were fixed. APOs were embedded in paraffin, serially sectioned at 5 μm, and mitotic figures were enumerated in Delfield’s hematoxylin and eosin-stained sections at 1000× magnification.

For elucidating the time course of the response, Salvador or NIH albino snails were injected with CBSS or 0.1 mg/ml LPS, a concentration found to be mitogenic for both strains of snails in the above dose-response study, followed by incubation in individual containers at 27 °C, as described above. At 18, 24, 48, or 72 h PI, the pericardial sac was fixed and processed for histological examination, without colchicine treatment. For each time period, 5 pericardial sacs from snails injected with CBSS or LPS were fixed. Additionally, pericardial saccs from 5 un.injected snails were examined.

2.4. In vitro response

The anterior pericardial wall was dissected from the pericardial sac of Salvador snails and exposed for 24 h to one of 7 concentrations of LPS, ranging from 0.0001–0.06 mg/ml, in a volume of 200 μl as previously described (Salamat and Sullivan, 2008). At each concentration, 6 APOs were examined histologically. Control APOs were incubated in CBSS only.

2.5. Statistical analysis

For the dose-response and in vitro studies, mean counts of mitotic figures at each LPS concentration were compared by one-way analysis of variance (ANOVA). Where significant differences were found, means were individually compared with the Student-Neuman–Keuls test using Statmost software (DataMost Corp, Salt Lake City, Utah). For the time course study, mean mitotic counts of matched CBSS- and LPS-injected snales were compared at each time interval with the two-tailed Student’s t-test.

3. Results and discussion

Although no effect was observed at 0.001 mg/ml, a significant increase in colchicine-arrested mitotic figures was observed at 24 h PI in APOs from Salvador snails injected with 0.01, 0.1, 1.0, and 10 mg/ml LPS, relative to CBSS-injected snales (Fig. 1). In as much as there were no statistically significant differences at concentra-
tions of 0.01–10 mg/ml, an increasing dose-response effect was not observed. In comparison to Salvador snails, numbers of mitotic figures in APOs of NIH albino snails were markedly lower at all concentrations (Fig. 1). Whereas hematopoietic tissue occupied a large region of the anterior pericardial wall in LPS-injected Salvador snails, typically only a few inactive follicles were observed in NIH albino snails, regardless of LPS dose (Fig. 2), and most dividing cells were scattered in loose connective tissue of the pericardial sac. Nevertheless, a statistically significant increase in mitotic activity occurred at concentrations of 0.1 mg/ml and above (Fig. 1). It was noted that snails injected with the highest concentration, 10 mg/ml, which contained visible particulate material, remained retracted in their shells for several hours PI, and usually failed to feed over the next 18 h, whereas at the lowest dose, snails emerged from their shells typically within 30 min PI and began feeding.

Among Salvador snails injected with 0.1 mg/ml and then fixed at different times PI, a significant increase in mitotic activity, relative to matched CBSS-injected snails, occurred at 18 h PI (Fig. 3).

This response peaked at 24 h PI, and was no longer statistically significant at 48 h PI. By 72 h PI, mitotic activity had returned to pre-injection levels. This pattern is similar to that observed with injections of freeze-thaw miracidial extract in a previous study (Sullivan et al., 2004), even attaining approximately the same peak value, although the response to miracidial extract was highest at 48 h PI rather than 24 h PI. This similarity suggests that there may be some overlap in the response mechanisms to two very different sources of non-self. For example, both types of stimuli may elicit the secretion of a common mitogenic cytokine, which then triggers an invariant mitotic response in the APO. This hypothetical mechanism would also account for the lack of a dose-response relationship (Fig. 1).

APOs of un.injected NIH albino snails showed a lower basal mitotic activity than that of Salvador snail APOs (Fig. 3), although the difference was not statistically significant. In contrast to APOs of Salvador snails, numbers of mitotic figures in APOs of NIH albino snails injected with CBSS or 0.1 mg/ml LPS were similar at all times PI. Possibly, the response in NIH albino snails is too weak to be detectable in the absence of colchicine treatment.

The low mitotic response to E. coli LPS in NIH albino B. glabrata is consistent with a previous study that found no response in these snails to LPS from Salmonella typhosa (Sullivan et al., 1984). These results raise the questions of whether NIH albino snails are consequently more susceptible to infection by Gram-negative bacteria, and whether this nonresponsiveness is linked to susceptibility to infection with larval schistosomes. Relative to the first question, we are not aware of any evidence showing increased frequency of Gram-negative bacterial infections in NIH albino snails, and in fact they survive injections with large numbers of viable Pseudomonas aeruginosa, Vibrio parahaemolyticus, and E. coli (Hanelt et al., 2008; Sullivan et al., 1984). Therefore, LPS-induced hematopoiesis may not play a significant role in the initial defense against bacterial invasions, which typically are rapidly eliminated by molluscs (Bayne, 1983). Concerning a possible association with schistosome susceptibility, penetration by miracidia of S. mansoni and injection with live E. coli elicit distinct transcriptional profiles in NIH albino B. glabrata at 12 h, suggesting that different mechanisms mediate early responses to the two types of challenge (Adema et al., 2010; Hanelt et al., 2008). Thus, the significance of the markedly different effects of LPS in NIH albino and Salvador B. glabrata remains to be determined.

Despite the strong response of Salvador APOs to LPS in vivo, APOs exposed in vitro to 0.0001–0.06 mg/ml, a range that fell within the hypothetical range of whole body concentrations in injected snails, showed no statistically significant increase in mitotic activity at any concentration (P > 0.05, ANOVA, data not shown). Since APOs are capable of responding in vitro to parasite extracts and to phorbol myristate acetate (Salamat and Sullivan, 2008, 2009), the lack of response to LPS may be evidence for the involvement of a peripheral tissue or hemolymph factor that is not present in vitro.

With regard to identifying the active component of crude LPS, it will be necessary to ascertain whether LPS itself and/or a co-extracted substance, e.g., PGN, triggers cell division, and if LPS, whether it is the lipid A or O-polysaccharide region of the molecule. Whereas lipid A is the only component of LPS that is recognized by the innate IDS of mammals (Miller et al., 2005), the majority of non-self receptors identified in molluscs are lectins (Bayne, 2009). Consequently, the O-polysaccharide of LPS is itself a candidate PAMP.

Increased cell division in the hematopoietic organ would seem a predictable response of an animal to exposure to PAMPs from a Gram-negative bacterium. However, no such proliferative response to bacterial PAMPs occurs in adult Drosophila, in which the hematopoietic lymph glands disappear at the pupal stage (Lemaître and Hoffmann, 2007); instead, they respond by secret-
ing antimicrobial peptides, as reviewed above. In mammals, LPS can induce cell division, e.g., by directly stimulating B cell proliferation (Anderson et al., 1972) or indirectly by inducing macrophages and other cell types to secrete mitogenic colony stimulating factors (Lee et al., 1990). However, this cell proliferation involves cells and TLR4-mediated signaling pathways that to our knowledge do not occur in C. glabrata. Thus, the increased cell division in the APO of B. glabrata following injection of LPS represents a novel systemic response of an invertebrate IDS to the PAMP(s) of Gram-negative bacteria.

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References


