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Activation of an innate immune response in the schistosome-transmitting snail *Biomphalaria glabrata* by specific bacterial PAMPs

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Abstract

Injection of crude lipopolysaccharide (LPS) from *Eschericia coli* into the hemocoel of *Biomphalaria glabrata* stimulates cell proliferation in the amebocyte-producing organ (APO). However, it is not known if mitogenic activity resides in the lipid A or O-polysaccharide component of LPS. Moreover, the possible role of substances that commonly contaminate crude LPS and that are known to stimulate innate immune responses in mammals, e.g., peptidoglycan (PGN), protein, or bacterial DNA, is unclear. Therefore, we tested the effects of the following injected substances on the snail APO: crude LPS, ultrapurified LPS (lacking lipoprotein contamination), two forms of lipid A, (diphosphoryl lipid A and Kdo₂-lipid A), O-polysaccharide, Gram negative PGN, both crude and ultrapurified (with and without endotoxin activity, respectively), Gram positive PGN, PGN components Tri-DAP and muramyl dipeptide, and bacterial DNA. Whereas crude LPS, ultrapurified LPS, and crude PGN were mitogenic, ultrapurified PGN was not. Moreover, LPS components, PGN components, and bacterial DNA were inactive. These results suggest that it is the intact LPS molecule which stimulates cell division in the APO.

Keywords

amebocyte-producing organ; mitotic cell division; molluscs; peptidoglycan; *Schistosoma*

1. Introduction

Pathogen associated molecular patterns (PAMPs) are conserved products of microbial metabolism that are recognized by pattern recognition receptors (PRRs) in multicellular organisms, leading to an innate immune response that in mammals involves secretion of costimulatory molecules, chemokines, and cytokines (Janeway and Medzhitov, 2002). Lipopolysaccharide (LPS) from Gram negative bacteria is a well characterized PAMP in mammals that also elicits a variety of innate immune responses in molluscs, including increased cell division among hemocyte precursors in the amebocyte-producing organ (APO) of *Biomphalaria glabrata*, the snail intermediate host of the human pathogenic blood fluke, *Schistosoma mansoni* (Sullivan et al., 2011). This mitotic burst in the APO, occurring at 18–24 h post-injection with crude (phenol-extracted) LPS, is of interest inasmuch as it

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represents a previously undescribed response of the molluscan internal defense system to bacterial PAMPs, and it resembles the reaction to incompatible larval trematodes or their injected molecules. Although the mechanism for this effect is not known, in mammals the cell surface PRR Toll-like receptor 4 (TLR4) mediates responses to LPS (Janeway and Medzhitov, 2002). Interestingly, Wang et al. (2011) have reported increased transcription of 5 genes encoding members of a putative TLR signaling pathway in the scallop *Chlamys farreri* following LPS exposure.

LPS makes up most of the outer leaflet of the outer membrane of the cell wall of Gram negative bacteria, and consists of 3 regions: lipid A, core, and O-polysaccharide or O-antigen [for detailed illustrations of cell wall components discussed here, see Esko et al. (2009), accessible online at <http://www.ncbi.nlm.nih.gov/books/NBK1945/>]. Lipid A is comprised of 6 saturated fatty acids attached to a phosphorylated n-acetylglucosamine (NAG) dimer, and anchors LPS in the membrane. The core is an oligosaccharide, with its inner region containing two unusual sugars not found in vertebrates, 1–4 residues of keto-3-deoxyoctononic acid (Kdo) joined to NAG of lipid A, and heptose. The O-polysaccharide joins to the outer region of the core and consists of a repeat unit of 1 to 8 sugars, repeated up to 50 times with an additional cap of 0–50 residues (Esko et al., 2009). Whereas the lipid A component of LPS, after first binding to a hydrophobic pocket in the coreceptor MD-2, is responsible for the TLR4-mediated innate immune response (Meng et al., 2010), the O-polysaccharide moiety elicits antibody production in mammals, resulting in more than 170 serotypes for *E. coli* alone (Esko et al., 2009). However, the O-polysaccharide is of interest as a potential PAMP in molluscs, in view of the role of carbohydrate-binding lectins in the internal defense system of these invertebrates (Sullivan et al., 2011).

A difficulty in interpreting reports of biological responses to crude LPS is that commercial preparations may be contaminated with other bacterial PAMPs that could independently elicit responses. Indeed, Hellman et al. (2003) pose the unsettling question of whether results of some of the thousands of studies of effects of LPS over the past several decades have been confounded by such contaminants. Among such substances are peptidoglycan (PGN), proteins, and DNA. PGN from *E. coli* consists of alternating N-acetylglucosamine and N-acetyl muramic acid residues arranged in strands of 25–35 disaccharide units that are cross-linked by short peptides that include meso diaminopimelic acid (DAP) (Esko et al., 2009). Release of antimicrobial peptides by the fat body of larvae of *Drosophila melanogaster* injected with crude LPS results not from LPS itself but rather from DAP-containing PGN that activates the IMD pathway (Kaneko et al., 2004), and Lemaitre and Hoffman (2007) have concluded that LPS does not activate Toll or IMD pathways in *Drosophila*. Analogously, trout macrophages, despite expressing cell surface TLR4, secrete cytokines when exposed to PGN but are unresponsive to LPS (MacKenzie et al., 2010). In mammals PGN fragments are detected by two types of endosomal PRRs, NOD1 and NOD2, members of the NLR family of proteins that have been found in a wide variety of multicellular organisms, including plants (Kaparakis et al., 2007). Human NOD1 detects the tripeptide L-alanine- γ -D-glutamate-meso-diaminopimelate (Tri-DAP), a component of all Gram negative and some Gram positive PGN, whereas NOD2 recognizes N-acetylmuramyl-L-alanyl-D-isoglutamine (muramyl dipeptide, MDP), a component of all bacterial PGN (Kaparakis et al., 2007). Lipoproteins, which number over 90 in *E. coli* (Kovaks-Simon et al., 2011), and outer membrane proteins, e.g., OmpA, are common contaminants of phenol-extracted LPS (Hellman et al., 2003). In mammals these proteins activate the TLR2 pathway (Kovaks-Simon et al., 2011; Pore et al., 2012). Finally, crude (phenol-extracted) LPS contains nucleic acid, mostly RNA but also DNA (Perdomo and Montero, 2006). Therefore, bacterial DNA, which has unmethylated CpG motifs that are recognized by TLR9 in mammals (Takeda and Akira, 2005), is also of interest as a possible APO mitogen in *B.*

glabrata, especially in light of the report by Hong et al. (2006) that injection of *E. coli* genomic DNA enhances hemocyte bactericidal activity in the mussel *Hyriopsis cumingii*.

Thus, the role of these and possibly other molecules in the mitotic response of *B. glabrata* to injected crude LPS remains to be determined. The goal of this study was to test individual components of LPS, i.e., lipid A and O-polysaccharide, as well as two additional candidate PAMPs that are potential contaminants of crude LPS, PGN and bacterial DNA, for their roles in triggering mitotic activity in the APO of *B. glabrata*.

2. Materials and Methods

2.1. Snails

The Salvador strain of *B. glabrata*, measuring 9.5 to 10.5 mm in shell diameter, was used for all experiments. Snails were reared in aerated aquaria containing artificial pond water at 25–27 °C and fed Romaine lettuce as described previously (Sullivan et al. 2011).

2.2. Chemicals

All chemicals were obtained from commercial sources and were injected at a concentration of 1 mg/ml, except as noted below for LPS from *E. coli* O111:B4 (1 and 10 mg/ml) and for diphosphoryl lipid A (0.1 mg/ml). A previous study had shown that crude LPS significantly enhanced mitotic activity in the APO at when injected at concentrations from 0.01 mg/ml to 10 mg/ml (Sullivan et al., 2011). Lipopolysaccharide concentrations are those provided by the supplier and are given below as endotoxin units (EU)/mg.

LPS and LPS components—LPS purified by phenol extraction from *E. coli* O127:B8 (LPS-B8, 5×10^5 EU/mg) (Sigma-Aldrich, St. Louis, MO) was dissolved in a 1/3 dilution of phosphate buffered saline (Kodak, Rochester, NY), hereafter referred to as PBS. Additionally, two other preparations of LPS were used, both from InvivoGen (San Diego, CA): LPS from *E. coli* O111:B4 (LPS-B4, 1×10^6 EU/mg) at both 1 and 10 mg/ml, and an ultrapure LPS from *E. coli* O111:B4 (LPS-B4U, 5×10^6 EU/mg), which lacks lipoprotein contaminants and is described by the supplier to only activate the TLR4 pathway, although possible PGN contamination is not specified. In order to identify an active component of LPS, 3 fractions were tested: diphosphoryl lipid A from *E. coli* F583 (Rd mutant) (Sigma Aldrich, St. Louis, MO), Kdo₂-lipid A (Avanti Polar Lipids, Alabaster, AL), and O-polysaccharide. Diphosphoryl lipid A, which is water insoluble, was first dissolved in dimethyl sulfoxide (DMSO) at its maximum concentration of 1 mg/ml. Because undiluted DMSO was toxic, causing 90% mortality among injected snails in preliminary studies, a 1/10 dilution of the stock solution in PBS (0.1 mg/ml) was injected. Kdo₂-lipid A was dispersed in PBS by sonication in a bath sonicator (Branson Ultrasonics Corp., Danbury, CT) prior to each injection, forming a homogeneous suspension. (Suspensions produced by vortexing alone invariably formed clumps that clogged the tip of the injection needle.) As described by Raetz et al. (2006), Kdo₂-lipid A is prepared from *E. coli* WBB06 (a heptose-deficient mutant, and hence lacking the remainder of the core and O-polysaccharide) and has higher endotoxin activity than lipid A itself, probably because of enhanced dispersal in aqueous media. O-polysaccharide was prepared from *E. coli* O127:B8 LPS by mild acid hydrolysis, according to the method of Shaw (1993). Briefly, LPS-B8 was added to 1% acetic acid and heated at 100 °C for 2.5 hr. The precipitated lipid was removed by centrifugation at 12,000 g for 20 min, and the O-polysaccharide-containing supernatant was lyophilized and resuspended in PBS. Since no further separation was carried out, this preparation presumably would also contain monosaccharides, core oligosaccharides, and isolated repeat units (Shaw, 1993).

PGN and PGN components—Three bacterial sources of PGN were used, all suspended in endotoxin-free H₂O (Invivogen, San Diego, CA): *E. coli* O111-B4 (PGN-EC) (10²–10³ EU/mg), *E. coli* K12 ultrapurified (PGN-ECU) (< 1 EU/mg), and the Gram positive species *Bacillus subtilis* (PGN-BS) (< 1 EU/mg), all from Invivogen. PGN samples were suspended by sonication in a bath sonicator prior to each injection. Additionally, the PGN agonists for NOD1 and NOD2, Tri-DAP (<0.125 EU/mg) and MDP (<0.125 EU/mg), respectively (Invivogen, San Diego, CA), were injected at a concentration of 1 mg/ml endotoxin-free H₂O.

DNA—Genomic DNA from *E. coli* K12 (< 1 EU/mg) (Invivogen, San Diego, CA) was dissolved in endotoxin-free H₂O.

2.3. Controls

Control injections were PBS (for crude LPS, Kdo₂-lipid A, and O-polysaccharide), 10% DMSO in PBS (for diphosphoryl lipid A), and endotoxin-free H₂O (for PGN and DNA).

2.4. Injection procedure and assessment of mitotic response

Volumes of 5 µl were injected through a hole in the shell and into a hemolymph sinus anterior to the digestive gland on the left side (see Sullivan, 1990). For each of the LPS samples 10 snails were injected, and for each of the PGN and DNA preparations 15 snails were used. Injected snails were incubated with food in individual 500-ml containers at 27 °C, and the pericardial sac was dissected and fixed in 1/3 Bouin's fluid, preheated to 50 °C, at 24 h post injection, the time at which the mitotic response to crude LPS peaks (Sullivan et al., 2011). Although snails in the earlier study were incubated in 0.1% colchicine for 6 h prior to fixation, we observed differences in extension of the head foot from the shell, locomotion, and feeding among snails injected with different substances. Therefore, due to concern that colchicine uptake would not be uniform across groups, this treatment was omitted. Mitotic figures in the anterior wall of the pericardial sac were enumerated in Delafield's hematoxylin and eosin-stained 5 or 7 µm-thick paraffin serial sections with the use of a 100x oil immersion objective.

2.5. Statistical analysis

Mean mitotic counts in APOs from snails injected with bacterial substances were compared with those from control snails injected with the same vehicle by means of the two-tailed Student's t-test, using Microsoft Excel. Data from snails injected with LPS (PBS or 10% DMSO controls) and those injected with cell wall components or DNA (H₂O controls) were analyzed separately. Probability values of <0.05 were considered statistically significant.

3. Results

Mitotic counts from snails injected with LPS and its components are shown in Figure 1. Mean counts for snails injected with crude LPS-B8, LPS-B4 (10 mg/ml) and LPS-B4U were significantly higher than those for PBS controls ($P = 0.003$, 0.027 , and 0.0006 , respectively), whereas LPS-B4 showed no activity at a concentration of 1 mg/ml. The tested components of LPS, i.e., diphosphoryl lipid A, Kdo₂-lipid A, and O-polysaccharide, did not induce a statistically significant increase in mitotic activity relative to their respective controls.

Mitotic counts from snails injected with cell wall components or bacterial DNA are shown in Figure 2. The mean for snails injected with PGN-EC was significantly higher than that for H₂O controls ($P = 0.03$). However, absence of endotoxin contamination (in PGN-ECU) also eliminated mitogenic activity. Likewise, Gram positive PGN (PGN-BS), PGN components (Tri-DAP and MDP) and *E. coli* DNA showed no activity.

4. Discussion

Results of this study support the previous observation that crude LPS from *E. coli* triggers an innate immune response in *B. glabrata*. Specifically, preparations of crude LPS from 2 strains of *E. coli* (O127:B8 and O111:B4) both elevated cell division in the APO, albeit only at 10 mg/ml for the second strain. Whether this difference between the two sources of crude LPS reflects an actual difference in the potency of LPS molecules themselves or results from differences in extraction techniques by the commercial suppliers is not known. Furthermore, LPS from which lipoprotein has been removed (LPS-B4U) was still mitogenic, suggesting that lipoproteins are not involved in this response.

Only the intact LPS molecule induced a response: DP-lipid A, Kdo₂-lipid A, and O-polysaccharide by themselves were inactive. These results are in contrast to the innate immune response to LPS in mammals, in which the lipid A moiety is responsible for endotoxin activity (Esko et al., 2009). Whether the O-polysaccharide is required in the *B. glabrata* response for binding to a PRR, or simply functions to enhance solubility, is not known. Another notable feature of the response in *B. glabrata* is that relatively high concentrations of LPS are required to induce cell division (Sullivan et al., 2011), versus the 2 ng/kg dose that elicits an innate immune response in humans in vivo (van Deventer et al., 1990) or the 100 ng/ml concentration that stimulates mouse splenic B cell proliferation in vitro (Xu et al., 2008). Thus, the mechanism by which LPS triggers cell division in the APO appears to be quite different from that involved in the mammalian innate immune response.

PGN did not stimulate cell division in the APO. Although relatively crude PGN (PGN-EC) was mitogenic, an ultrapure preparation of PGN that lacked endotoxin contamination (PGN-ECU) was inactive, suggesting that the LPS in PGN-EC was responsible for the observed response. Unlike LPS, which shows variability in its lipid and polysaccharide components among different bacteria (see Raetz and Whitfield, 2002), PGN structure is quite uniform among Gram negative bacteria (Schleifer and Kandler, 1972), and it is unlikely that the different strains of *E. coli* used as the sources of the crude and ultrapurified PGNs (O111-B4 and K12, respectively) explain the results.

We note two caveats. First, although PGN, lipoprotein, and bacterial DNA do not appear to directly stimulate cell division in the APO, we cannot rule out a possible enhancing effect of these and other substances on LPS activity, as has been shown for MDP in a guinea pig-endotoxic shock model (Ribi et al., 1979) and CpG-containing synthetic oligodeoxynucleotides in TNF- α secretion by mouse macrophages (Gao et al., 2001). Moreover, inasmuch as we examined only a single type of histological reaction in one anatomical region, we have no information on whether other types of snail innate immune responses are being stimulated by crude LPS, as seems likely based on the transcriptomic profiles in whole snails challenged with *E. coli* (Adema et al., 2010). Specifically, injection of *B. glabrata* with *E. coli* resulted in up-regulation of 83 mostly stress and immune-related transcripts at 12 h post injection.

In conclusion, we have attempted to ascertain whether LPS or a contaminant is responsible for the observed innate immune response in the APO of *B. glabrata* challenged with crude LPS, and if LPS itself, which component of LPS is active. By using commercially available bacterial products known to trigger innate immune responses in mammals, we have found that a purified form of LPS from which lipoprotein has been removed stimulates cell division, whereas isolated components of LPS, i.e., diphosphoryl lipid A, Kdo₂-lipid A, and O-polysaccharide, do not. Although PGN contaminated with LPS stimulates cell division, purified PGN, Gram positive PGN, as well as PGN components Tri-DAP and MDP, are all inactive. Another potential contaminant of crude LPS, i.e., DNA, similarly has no effect. On

the basis of these results, we conclude that it is the intact LPS molecule, and not components thereof or potential contaminants, that elicits cell proliferation in the APO of *B. glabrata*. The mechanism by which intact LPS is recognized by the snail's innate immune system remains to be determined.

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Abbreviations

APO	amebocyte-producing organ
DMSO	dimethyl sulfoxide
EU	endotoxin unit
Kdo	keto-3-deoxyoctonoic acid
LPS	lipopolysaccharide
LPS-B4	LPS from <i>Escherichia coli</i> O111:B4
LPS-B4U	LPS-B4, ultrapurified
LPS-B8	LPS from <i>Escherichia coli</i> O127:B8
MDP	muramyl dipeptide
NAG	n-acetylglucoseamine
PAMP	pathogen associated molecular pattern
PBS	1/3 dilution of phosphate buffered saline
PGN	peptidoglycan
PGN-BS	PGN from <i>Bacillus subtilis</i>
PGN-EC	PGN from <i>E. coli</i> O111-B4
PGN-ECU	PGN from <i>E. coli</i> K12, ultrapurified
PRR	pattern recognition receptor
TLR	Toll-like receptor
Tri-DAP	L-alanine- γ D-glutamate-meso-diaminopimelate

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- We investigate mitogenic activity of bacterial PAMPs in snails.
- LPS from two strains of *Escherichia coli* is mitogenic for hematopietic cells.
- Components of LPS, i.e., lipid A and O-polysaccharide, are inactive.
- Peptidoglycan and its components, as well as bacterial DNA, are also inactive.
- Only intact LPS stimulates cell division in the snail.

Fig. 1.

Mean number of mitotic figures/APO in snails injected with LPS, LPS components, or vehicle (PBS, 10% DMSO). Error bars represent standard errors of the mean. N = 10 snails/treatment. PBS, 1/3 phosphate buffered saline; 10% DMSO, 10% dimethyl sulfoxide in PBS; LPS-B8-1, crude LPS from *E. coli* O111:B8, 1 mg/ml PBS; LPS-B4-1, LPS-B4-10, crude LPS from *E. coli* O111:B4, 1 mg/ml and 10 mg/ml PBS; LPS-B4U-1, ultrapurified LPS-B4, 1 mg/ml PBS; DPLIPA-0.1, diphosphoryl lipid A, 0.1 mg/ml in 10% DMSO; KDOLIPA-1, keto-3-deoxyoctononic acid lipid A, 1 mg/ml PBS; O-POLY, O-polysaccharide, 1 mg/ml PBS. *, $P < 0.05$ vs. vehicle control, Student's t-test.

Fig. 2.

Mean number of mitotic figures/APO in snails injected with 1 mg/ml cell wall components, bacterial DNA or vehicle (endotoxin-free H₂O). Error bars represent standard errors of the mean. N = 15 snails/treatment. H₂O, endotoxin-free H₂O; PGN-EC, crude peptidoglycan from *Escherichia coli* O111-B4; PGN-ECU, ultrapurified PGN from *E. coli* K12; PGN-BS, crude PGN from *Bacillus subtilis*; MDP, muramyl dipeptide; TRI DAP, L-alanine- γ -D-glutamate-meso-diaminopimelate; EC DNA, DNA from *E. coli* K12. *, $P < 0.05$ vs. vehicle control, Student's t-test.