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The lipopolysaccharide (LPS) of *Photorhabdus luminescens* TT01 can elicit dose- and time-dependent immune priming in *Galleria mellonella* larvae

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ABSTRACT

In this work, we primed *Galleria mellonella* larvae by haemocoel injection of lipopolysaccharide (LPS) extracted from *Photorhabdus luminescens* TT01 to determine whether bacterial LPS can induce enhanced immune protection (recently called immune priming). We also analyzed the relationship between changes in the levels of innate immune elements and the degree of enhanced immune protection in the larvae at designated time points after priming. The larvae that received experimental doses (20.0, 10.0 and 5.0 μg per larva) of LPS demonstrated increased resistance against lethal challenge with *P. luminescens* TT01; the degree and period of protection correlated positively with the priming dose. These results indicated that the *P. luminescens* TT01 LPS could induce typical immune priming in *G. mellonella*. Moreover, the levels of innate immune parameters (i.e. haemocyte density, phagocytosis, haemocyte encapsulation ability, and antibacterial activity of cell-free haemolymph) and endogenous enzyme activities (i.e. acid phosphatase, ACP; alkaline phosphatase, AKP; superoxide dismutase, SOD and lysozyme, LSZ) were significantly increased following priming of the larvae with LPS, whereas the activities of peroxidase (POD) and catalase (CAT) were significantly decreased. All of the parameters examined changed in a dose- and time-dependent manner. This study demonstrated that *G. mellonella* larvae could modulate their immune responses based on different doses of LPS used for priming, and that priming phenomenon in *G. mellonella* larvae elicited by LPS was mediated by the innate immune elements and enzyme activity.

1. Introduction

The immune system of invertebrates is presumed to lack memory and specificity because invertebrates lack the lymphocytes and functional antibodies that form the basis of the adaptive immune system of vertebrates (Janeway and Medzhitov, 2002). However, several recent studies have shaken this paradigm and suggested that the immune defenses of invertebrates are more complex and specific than previously thought. For instance, primary exposure of invertebrates to pathogens increased their resistance to a later pathogenic challenge. This mechanism, termed immune priming, has been reported in animals such as *Bombus terrestris* (Sadd and Schmid-Hempel, 2006), *Drosophila Melanogaster* (Pham et al., 2007) and *Tribolium castaneum* (Roth et al., 2009). Our recent work demonstrated that priming *Galleria mellonella* larvae with an appropriate dose of heat-killed bacterial cells by haemocoel injection resulted in a significant increase in larval resistance against a lethal challenge with viable *Photorhabdus luminescens* TT01. Thus, *G. mellonella* larvae possess typical immune priming ability (Wu et al., 2014). The mechanism underlying priming in *G. mellonella* is sophisticated; therefore, whether the cell wall component of bacteria can induce a strong immune priming response is unclear.

P. luminescens TT01 is the Gram-negative symbiotic bacteria of the entomopathogenic nematode *Heterorhabditis bacteriophora* which is highly pathogenic to many insects. The *P. luminescens* TT01 genome contains numerous genes that are predicted to encode toxins, hemolysins, and proteases that may contribute to insect pathogenicity (Duchaud et al., 2003). LPS are amphiphilic molecules that are localized in the outer leaflet of the outer

Abbreviations: LPS, Lipopolysaccharide; AMPs, antimicrobial peptides; SOD, superoxide dismutase; POD, peroxidase; CAT, catalase; ROS, phenoloxidase; LSZ, lysozyme; ACP, acid phosphatase; AKP, alkaline phosphatase; PG, peptidoglycan; PTU, phenylthiourea; FITC, fluorescein isothiocyanate; GIM, Grace's insect medium; PGRP, peptidoglycan receptor protein; PRRs, pattern recognition receptors.

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membranes of Gram-negative bacteria. These molecules are highly antigenic and have endotoxic properties. For example, the lipid A moiety of LPS has been demonstrated to stimulate the TLR4 signaling pathway in mammals (Meng et al., 2010). LPS are able to stimulate the expression of antimicrobial peptides (AMPs) in the haemocytes and fat bodies of *Manduca sexta* larvae in a dose-dependent manner (Rao and Yu, 2010), LPS is also a potent elicitor of the activation of immune-related genes in several other insect species, including *D. melanogaster*, *T. castaneum* and *Bombyx mori* (Altincicek et al., 2008; Ha Lee et al., 2007; Jin et al., 2008). Therefore, demonstrating the existence of an LPS induced priming response in *G. mellonella* and further assessing the relationship between the LPS dose required for priming and the degree of the elicited immune response are important factors for explorations of the underlying mechanisms of immune priming in invertebrates.

The immune defense system of insects relies on several innate humoral and cellular responses that are tightly interconnected. The humoral element of the immune response consists of proteins and antimicrobial peptides that are capable of killing invading microorganisms (Lowenberger, 2001; Mak et al., 2010; Nelson et al., 2013). The cellular immune responses consists of haemocytes that possess functions related to the phagocytosis and encapsulation of invading cells, and nodule formation around larger structures (Grizanova et al., 2014; Satyavathi et al., 2014). Haemocyte functions have a close connection with the endogenous enzymes that are excreted and released by haemocytes via exocytosis and degranulation. For example, acid phosphatase (ACP) and alkaline phosphatase (AKP) have been found in insect haemocytes; their release into the plasma can enhance the phagocytic activity of haemocytes (Cheng and Butler, 1979; Xia et al., 2000). Superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT) are able to eliminate excess reactive oxygen species (ROS); ROS play an important role in oxidative killing against pathogens, but are also toxic to the hosts (Ahmad, 1995). Lysosomes have been found within the granular haemocytes of many invertebrates and are thought to be one of the primary enzymes produced by haemocytes, This enzyme covers a wide antibacterial spectrum and exhibits high activity against Gram-positive bacteria and some Gram-negative bacteria (Yu et al., 2002a). However, to the best of our knowledge few studies have been carried out to determine the functions of endogenous enzymes in the immune priming responses of *G. mellonella* larvae.

The aim of this work was to determine whether LPS can induce a priming response against *P. luminescens* TT01 challenge in *G. mellonella* larvae, and to characterize the changes in immune parameters and endogenous enzyme activities of the larval haemolymph in the priming response. Furthermore, we also analyzed the functions of immune elements and enzymes involved in the priming process.

2. Materials and methods

2.1. Bacteria and insect

The bacteria *P. luminescens* TT01 used in this study were preserved in liquid nitrogen in the Bacterial and Nematode Collection of SKLB, Sun Yat-sen University. *P. luminescens* TT01 were streaked onto an NBTA (nutrient bromothymol blue-triphenyl tetrazolium chloride agar) plate. Then, a primary-form colony was picked, inoculated into LB broth and cultured at 28 °C on a shaker to an OD_{600} of approximately 1.5.

G. mellonella (Lepidoptera, Pyralidae) larvae were reared on an artificial diet (22% maize meal, 22% wheat germ, 11% dry yeast, 17.5% bee wax, 11% honey and 11% glycerin) at 28 °C in the dark. Early fifth instar *G. mellonella* larvae with a weight of approximate 250 mg were chosen for all experiments.

2.2. Preparing LPS from P. luminescens TT01

The *P. luminescens* TT01 cells used for LPS extraction were harvested and washed three times with sterilized phosphate buffered saline (PBS: 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4 and 0.24 g KH_2PO_4 in 1000 ml distilled water, pH 7.2) by centrifugation. LPS was extracted using a commercial LPS Extraction Kit (catalog: 17141) (iNtRON, Korea) according to the manufacturer's instructions. To eliminate contaminating proteins and nucleic acids, proteinase K (100 μg/ml) (Sigma) was added to the LPS mixture, and the tubes were incubated at 65 °C for one hour. The mixture was subsequently treated with RNase (40 μg/ml) (Sigma) and DNase (20 μg/ml) (Sigma) in the presence of 1 μl/ml 20% $MgSO_4$ and 4 μl/ml chloroform and the incubation was continued at 37 °C overnight. Subsequently, the mixture was centrifuged at 13,000*g* for 3 min, and then the LPS was rinsed with 70% ethanol. The sample was incubated at −20 °C overnight to precipitate the LPS. The precipitate was centrifuged at 2000*g* at 4 °C for 10 min, and then the supernatant was discarded. The precipitate was dried and resuspended in PBS buffer.

2.3. Tolerance of G. mellonella larvae to haemocoel injection of LPS extracted from P. luminescens TT01

All haemocoel injections used the following protocol: Prior to injection, each larva was surface sterilized with 70% ethanol. A 50 μl Hamilton syringe was used to inject 10 μl of PBS containing a designated amount of LPS into the haemocoel of each larva via the last left pro-leg. After injection, the larvae were reared under the same conditions, and their behaviors and mortality were recorded.

To determine an appropriate priming dose, 10 μl of PBS containing 80.0, 60.0, 40.0, 20.0 or 0 μg (the control) of LPS was injected into the haemocoel of each larva. Mortality was recorded every 12 h for 5 days. For each treatment, twenty-one larvae were injected and three independent trials were conducted.

2.4. Effect of different LPS priming doses on the immune protection level

Seven groups of *G. mellonella* larvae were immune-primed by haemocoel injection of 10 μl of PBS containing 40.0, 20.0, 10.0, 5.0, 1.0, 0.5 or 0 μg (the control) of LPS per larva. Twenty-four hours after immune-priming, each larva was injected with 10 μl of PBS solution containing approximately 50 viable *P. luminescens* TT01. The numbers of injected bacteria were estimated using a haemocytometer and confirmed by plating a known volume of the injected suspension on NBTA plates. Mortality was recorded 72 h after challenge. For each treatment, fifteen larvae were tested and three independent trials were conducted.

2.5. Dose- interval time-dependent immune priming assay

Four groups (100 larvae per group) of *G. mellonella* larvae were immune-primed by haemocoel injection of 10 μl of PBS containing 20.0, 10.0, 5.0 or 0 μg (the control) of LPS per larva. At 0, 12, 24, 72, 168 and 336 h after immune-priming, fifteen larvae were sampled from each group. Each larva was infected by injection with 10 μl of a PBS suspension containing approximately 50 viable *P. luminescens* TT01 cells. Mortality was scored 72 h after challenge. Three independent trials were performed for all treatments.