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Effects of dietary heavy metals on the immune and antioxidant systems of *Galleria mellonella* larvae

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ABSTRACT

In this work, we analyzed the effects of chromium (Cr) and lead (Pb) on immune and antioxidant systems of *Galleria mellonella*. In particular, after exposure to diets containing environmentally relevant concentrations (5, 50 and 100 μg/g) of Cr or Pb for 7 d, alterations in innate immune parameters and the activity of endogenous enzymes were measured in larvae. The results showed that 1) compared with the control, the lowest doses (5 μg/g) of Cr and Pb significantly increased the levels of innate immune parameters (total hemocyte count, THC; phagocytic activity; extent of encapsulation) of the larvae and hemolymph immune enzyme activities (acid phosphatase, ACP; alkaline phosphatase, AKP; phenoloxidase, PO), whereas the highest doses (100 μg/g) of Cr and Pb inhibited them; 2) the activity of antioxidant enzymes (superoxide dismutase, SOD; peroxidase, POD; catalase, CAT) showed significant increases with increasing concentrations of dietary Cr and Pb, and were significantly higher than those of the control; and 3) feeding the larvae with experimental concentrations of either Cr or Pb resulted similar patterns of changes of all the parameters examined. The current study suggested that moderate amounts of Cr and Pb enhance the innate immunity of *G. mellonella*, but that large amounts led to the inhibition of larval immune function, and also indicated that the experimental concentrations of Cr and Pb used caused strong oxidative stresses in the larvae.

1. Introduction

Emissions of heavy metals into the environment occur via a wide range of processes, including input to soil, surface waters and air (Jarup, 2003). Heavy metal pollution can have harmful effects not only on the survival, growth, reproduction and metabolism of animals but also on the innate immune system. Indeed, a great deal of effort has been directed toward elucidating the possible correlation between heavy metal pollution and stress-related disease conditions in animals (Galloway and Depledge, 2001; Dubovskiy et al., 2011). It is known that the innate immune system of invertebrates, which plays a critical role in protecting the body from infection, shares a high degree of homology with that of mammals (Salzet, 2001). Therefore, many invertebrates, including insects, have been postulated as good models for studying the toxicity of heavy metals and as ideal indicator organisms for assessing levels of environmental pollution.

The immune systems of insects include both cellular and humoral defense responses, and circulating hemocytes are part of the defense against potential pathogens (Lavine and Strand, 2002). Endogenous enzymes excreted and released by hemocytes by exocytosis and degranulation have a close connection with the immune functions of hemocytes. For example, superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT), three typical antioxidases, are able to eliminate excess reactive oxygen species (ROS) which play an important role in oxidative killing against pathogens, but are also toxic to hosts (Felton and Summers, 1995; Wang et al., 2001). Activated hemocytes can undergo an "oxidative burst" by releasing ROS. ROS include oxygen ions, free radicals and peroxides, both inorganic and organic (Lee et al., 2012). These molecules are generally very small and are highly reactive, due to the presence of unpaired valence shell electrons. ROS play an important role in cell signaling and the induction of host defense genes (Dalton et al., 1999). Recent investigations of the invertebrate immune system have found that ROS dependent immunity is important for survival (Ha et al., 2005). While "oxidative burst" is effective against pathogens, it can also result in damage to tissues when ROS production is enhanced to a greater level due to an imbalance between oxidant and antioxidant status in the cell called "oxidative stress". The antioxidases are able to eliminate excessive ROS to alleviate oxidative damage to hosts. Acid phosphatase (ACP) and alkaline phosphatase (AKP), two typical hydrolases that have been found in insect hemocytes and shown to be released into the plasma, can enhance the phagocytosis responses of hemocytes by modification of the surface molecular structure of pathogens (Cheng and Butler, 1979; Xia et al., 2000).

Abbreviations: SOD, superoxide dismutase; POD, peroxidase; CAT, catalase; ROS, reactive oxygen species; PO, phenoloxidase; ACP, acid phosphatase; AKP, alkaline phosphatase; THC, total hemocyte count; PTU, phenylthiourea; FITC, fluorescein isothiocyanate; GIM, Grace's insect medium.

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Phenoloxidase (PO), which has a number of important roles in insect immunity including wound healing, melanization and encapsulation, is often used as an indicator of the immunocompetence of individuals (Lemaitre and Hoffmann, 2007).

Both phagocytosis and encapsulation are the main components of the insect cellular response (Lavine and Strand, 2002). Phagocytosis refers to the engulfment of targets, such as bacteria or yeast, by an individual hemocyte and encapsulation results in the binding of hemocytes with larger targets, such as parasitoids and nematodes. In a previous study, we demonstrated that both granular cells and plasmatocytes in the hemolymph of *Galleria mellonella* phagocytosed FITC-labeled bacteria in vitro (Wu et al., 2014). During an encapsulation response, granular cells contacted the invaders first and then released material that promoted the attachment of plasmatocytes, eventually forming a smooth capsule comprised of overlapping layers of cells (Schmit and Ratcliffe, 1977; Pech and Strand, 1996). The total hemocyte count can be affected by changes in environmental conditions, and different types of stressors, including pathogens, parasites and xenobiotics, have been identified as hemocyte effectors (Bergin et al., 2003). The cellular immune response patterns mediated by hemocytes may depend on the environmental stressor and could therefore be used as biomarkers to discriminate between biological and chemical alterations in the environment.

During the past decade, the cellular and humoral immune parameters of invertebrates have been recognized as important tools in the evaluation of the physiological condition of organisms and the level of environmental pollution. However, previous studies that examine the effects of heavy metal pollution on invertebrate immune function were largely conducted using mollusks (Duchemin et al., 2008; Mottin et al., 2010), crustaceans and oligochaetes (Sun and Zhou, 2008; Singaram et al., 2013). In contrast, the effects of pollution on immunological defense mechanisms have been poorly investigated in insects. Both Cr and Pb are ubiquitous, cumulative and insidious environmental toxins that induce a broad range of physiological, biochemical, and behavioral dysfunctions (Alcutt and Pinto, 1994; Lee et al., 2012). Therefore, a study of the effects of chromium (Cr) and lead (Pb) on immune and the antioxidant systems of *G. mellonella* was conducted.

The objective of the present study was to determine whether a 7 d exposure to environmentally relevant concentrations of Cr and Pb would induce physiological changes in the immune system and to determine the response of the antioxidant system. Specifically, changes in immune parameters such as total hemocyte count (THC), phagocytic activity and extent of encapsulation were measured. In addition, changes in the activities of endogenous enzymes, such as ACP, AKP, PO, SOD, POD and CAT were quantified in the cell-free hemolymph. The present study is a first attempt at understanding the effects of Cr and Pb on the immune and antioxidant systems of *G. mellonella* exposed to Cr or Pb.

2. Materials and methods

2.1. Insects and heavy metal treatment

G. mellonella larvae were collectively reared in glass containers at 28 °C in the dark and initially fed an artificial diet (22% maize meal, 22% wheat germ, 11% dry yeast, 17.5% bee wax, 11% honey and 11% glycerin). Once the larvae reached their fourth instar they were conducted a 24 h starvation treatment for the following experiment.

We prepared a series of experimental diets: one diet (30 g artificial diet) was added 5 ml of distilled H_2O (control). The second diet (30 g artificial diets per treatment group) was added an experimental solution (5 ml per experimental diet), instead of distilled water, containing different concentrations of $CrCl_3 \cdot 6H_2O$ (153.8 μg/ml, 1538.0 μg/ml or 3076.0 μg/ml) or $Pb(NO_3)_2$ (48.0 μg/ml, 480.0 μg/ml or 960.0 μg/ml). All the experimental diets were placed in electric blast drying oven for 6 h at 30 °C and total weight of each experimental diet was maintained to about 30 g. The experimental larvae (30 larvae per treatment group) were exposed to dietary Cr or Pb at final concentrations of 0 (control), 5 μg/g, 50 μg/g and 100 μg/g, respectively.

These concentrations of Cr or Pb had no effect on the mortality of the larvae. All the larvae from each group were collected following by a 7 d period of feeding, and used for hemolymph collection to examine immune parameters and enzymes activities. For all treatments, three independent trials were performed.

2.2. Hemolymph sample preparation

In each group, 40 μl of fresh hemolymph from each larva was collected by pricking the larva with an insect needle; the hemolymph was stored in micro-tubes on ice to minimize cell clumping. The hemolymph from each individual was divided into several aliquots and processed as follows: 1) 20 μl of the hemolymph was diluted in five volumes of ice-cold anticoagulant solution (93 mM NaCl, 100 mM glucose, 30 mM trisodium citrate, 26 mM citric acid, 10 mM Na_2EDTA, and a few crystals of phenylthiourea (PTU), pH 4.6.) and used immediately for total hemocyte count; 2) 10 μl of the hemolymph was immediately added to 20 μl ice-cold anticoagulant buffer for phagocytosis assays; 3) 30 μl of hemolymph was collected for encapsulation assays; 4). The remaining hemolymph was immediately transferred into sterile chilled Eppendorf tubes and centrifuged at 500 ×*g* for 5 min at 4 °C to pellet the cellular debris. The hemolymph plasma samples were stored at −80 °C for later measurement of enzyme activities.

2.3. Hemocyte count

To determine the total hemocyte count (THC), aliquots of the hemolymph suspension obtained as described above were diluted and transferred to a Neubauer hemocytometer. The total hemocyte number was counted using a phase contrast microscope.

2.4. In vitro phagocytosis assay using FITC-labeled bacteria

Phagocytosis tests were performed using hemocyte monolayers with dead *Photorhabdus luminescens* TT01 bacteria as the substrate. The fluorescent bacteria were prepared as previously described (Wu et al., 2014). Briefly, TT01 was cultured in Luria–Bertani (LB) broth at 28 °C with shaking at 200 rpm to an OD600 of approximately 1.5. The bacterial culture was heated for 20 min at 100 °C and then washed three times by centrifuging (1300 ×*g*, 4 °C, 10 min) with filter-sterilized (0.22 μm) 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). The heat-killed TT01 cells were resuspended in carbonate buffer (0.2 M Na_2CO_3, 0.2 M $NaHCO_3$, pH 9.4) containing fluorescein isothiocyanate (FITC, 0.1 mg/ml). The suspension was then incubated for 30 min in the dark at 28 °C and 200 rpm on a rotary mixer, washed 3 times with PBS to remove all traces of free FITC and resuspended in Grace's insect medium (GIM) at 10^9 cells/ml. The bacterial suspension was vortexed thoroughly to eliminate clumping and then stored at −20 °C.

The in vitro phagocytosis quenching assay was performed according to the method described by Asgari and Schmidt (Asgari and Schmidt, 2003) with the following changes: an aliquot of 30 μl of the hemolymph-anticoagulant suspension was added to a well of 24-well cell culture plate attached with a 12-mm-diameter round glass coverslip prefilled with 300 μl of ice-cold Grace's insect medium (GIM) and incubated at 28 °C for 30 min in the dark. The samples were washed three times with GIM to remove the anticoagulant buffer. Immediately after washing, 20 μl of FITC-labeled bacterial suspension in GIM solution and 380 μl GIM were added to each well and incubated for 2 h in darkness at 28 °C. Afterwards, 300 μl of 0.4% trypan blue solution in GIM was placed onto each coverslip after removal of the culture medium. After incubation for 20 min, the coverslips were washed three times with GIM and then treated with 4% formaldehyde for 30 s to fix