Contributions of cellular and humoral immunity of *Galleria mellonella* larvae in defence against oral infection by *Bacillus thuringiensis*

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*Article info*

**Abstract**

In this study the cellular and humoral immune reactions of the Greater wax moth *Galleria mellonella* have been investigated during bacterial infection caused by oral administration of *Bacillus thuringiensis*. Two different dose strengths were investigated to assess the contribution of immune parameters to induced Bt resistance. Low-dose (sublethal LC₅₀) infection resulted in significantly elevated haemolymph phenoloxidase and lysozyme-like activity, enhanced phagocytic activity of haemocytes, and increased encapsulation responses in infected larvae at 48 and 72 h post infection. Higher doses of Bt (half-lethal LC₅₀) also triggered significantly elevated haemolymph phenoloxidase and lysozyme-like activity, but decreased the coagulation index and activity of phenoloxidase in haemocytes of infected larvae. In both types of infection, the pool of circulating haemocytes became depleted. The importance of cellular and humoral immune reactions in induced insect resistance to intestinal bacterial infection Bt is herein discussed.

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**Keywords:**

Phagocytosis

Encapsulation

PO

Resistance

Insects immunity

1. Introduction

The spore-forming Gram positive bacterium *Bacillus thuringiensis* Berliner (Bt) is widespread in nature, being present in soil, leaf litter and in microflora on the surface of leaves (Aptosoglu et al., 1997; Chak et al., 1994; Chilcott and Wigley, 1993; Smith and Couche, 1991). Bt is a pathogenic agent for many insect orders, acting via the synthesis of a highly diverse range of toxins and derivative metabolites (Nielsen-LeRoux et al., 2012; Raymond et al., 2010). The most common toxin of Bt is a crystalline protein δ-endotoxin that is synthesized during sporulation inside the vegetative cells, which also accommodate the spore. Upon ingestion, crystalline δ-endotoxin is first solubilised in the insect midgut. The gut protease fluids then process the pro-toxin into its mature, active form which will then cross the peritrophic matrix and bind to receptors on the brush border membrane of the gut epithelial cells (reviewed by Pigott and Ellar (2007)). Binding to receptors such as cadherins or aminopeptidase N triggers the formation of toxin oligomers that insert into the cell membrane, creating pores that cause osmotic cell shock (reviewed in Bravo et al. (2005) and de Maagd et al. (2003)). Alternative cell death pathways can also be triggered by receptor-cry binding (Higuchi et al., 2007; Zhang et al., 2006). The resulting epithelial cell death destabilizes and compromises the integrity of the gut epithelium, but the actual cause of Bt-mediated insect death is still a matter of some debate. It was originally thought that *B. thuringiensis* acts as a septicemic agent, but this does not explain why insects die when only the cell-free toxin is administered (Bravo et al., 2005). It is now recognised that other members of the gut microflora can cause septicemia (Broderick et al., 2006) however their role in the insecticidal activity of Bt is not obligatory (van Frankenhuysen et al., 2010).

The mode of Bt-mediated killing obviously has important implications for any potential tolerance strategies mounted by the insect. Resistance to Bt appears to be multifaceted, perhaps on a species-specific level: the midgut environment may not support the processing of the immature pro-toxin (Oppert et al., 1994), stem cell production in the gut lining may be elevated to replace lost epithelial cells (Martinez-Ramirez et al., 1999), and epithelial cell receptors may be inactivated or lost (e.g. Darboux et al., 2002; Gahan et al., 2001). A form of “passive” tolerance comprising competition from other gut bacteria is another (as yet unexplored) possibility, but it is already known that aggressive competition between Bt strains can result in reduced virulence (Garbutt et al., 2011). Of paramount important to the current study, however, is the notion that the insect immune system is a critical factor assisting tolerance to Bt, but the extent of the involvement of the immune response has yet to be properly explored.

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http://dx.doi.org/10.1016/j.jip.2014.04.003
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The natural mode of infection of insects by Bt is via the ingestion of contaminated food (Vallet-Gely et al., 2008). The insect immune response under per os infection (natural infection) differs both in the magnitude and localisation in comparison with aseptic penetration of bacteria (Basset et al., 2000; Vallet-Gely et al., 2008; Wang et al., 2010). Previous studies have been limited to showing that (i) haemolymph melanization is enhanced and melanization is also higher in the peritrictic matrix and surface of epithelial cells (Rahman et al., 2004), (ii) a haemolymph hexamerin coagulation factor is secreted into the gut lumen that can bind to and inactivates the cry toxin (Ma et al., 2005) and (iii) chemical or biological immunosuppression can both enhance the susceptibility of insects, and reduce recruitment of the insect’s resistance to intestinal Bt infections (Broderick et al., 2010; Kwon and Kim, 2007; Richards and Dani, 2010; Shrestha et al., 2010). Previously we have reported that sublethal doses of Bt enhance both the phagocytic and encapsulation activities of the Galleria mellonella larval haemolymph (Dubovskyi et al., 2008). However, these studies were preliminary and therefore limited in the range of immune responses and challenges assessed. There is much still to be understood about the involvement of the cellular immune response as well as other humoral responses, and it is relevant because this could constitute one mechanism by which insects evolve tolerance against low-dose pre-exposure to Bt (Ericsson et al., 2009; Rahman et al., 2004).

In nature, insects come into contact with both high and sublethal doses of bacteria. They are exposed to high doses during epizootic processes in natural conditions, or during Bt biopesticide treatment. At the same time, insects have permanent contact with sublethal doses of Bt contained naturally in the environment (soil, leaves, and rhizosphere) (Aptosoglu et al., 1997; Chak et al., 1994; Chilcott and Wigley, 1993; Smith and Couche, 1991). Thus, comparative analyses of insects’ immune responses under varying degrees of pressure exerted by Bt, could highlight key defence reactions and indicate which strategies are employed under different natural conditions, and also indicate possible costs associated with tolerance/resistance. Understanding the major defence responses of insects to Bt will help us to detect and/or predict the emergence of inducible resistance mechanisms that allow insect populations to survive and evolve under pressure from bacteria in biocenosis and agrocenosis (Ericsson et al., 2009; Hernandez-Martinez et al., 2010; Rahman et al., 2004).

In this study, a comprehensive range of cellular and humoral immune reactions in G. mellonella has been investigated during sublethal and half-lethal natural bacterial infection by B. thuringiensis, in order to gauge the protective role of immunity in insects exposed to differing intensities of intestinal infections.

2. Materials and methods

2.1. Chemicals

Phenylmethanesulfonylfluoride (PMSF), 3,4-dihydroxy- l-phenylalanine (l-DOPA), Fluorescein Isothiocyanate (FITC), Amaranth red, lipopolysaccharide (LPS), freeze-dried Micrococcus lysodeikticus, egg white lysozyme (EWL), bovine serum albumin (BSA) and phenylthiourea (PTU) were all supplied by Sigma–Aldrich, USA.

2.2. Insects and bacteria

The larvae of the Greater wax moth G. mellonella L. (Lepidoptera, Pyralidae) were derived from a long-established laboratory population. Insects were collectively reared in glass containers (0.7 l) at 28 °C in the dark, and initially fed an artificial diet containing bee honey, glycerol, beeswax and wheat flour. Fourth instar larvae were used for the experiments.

The bacterium B. thuringiensis ssp. galleria (H-serotype V) strain 69-6 used in the experiments was taken from the bacterial collection of the Institute of Systematics and Ecology of Animals (ISEA Siberian Branch Russian Academy of Sciences). A spore–crystal mixture of the bacterium was used for the peroral ingestion by insects. Sublethal LC15 and half-lethal LC50 doses were determined in a pilot study by giving a separate group of larvae five increasing doses of bacteria and then selecting the dose that came closest to killing 15% of the larvae (equating to 5 × 10^6 per ml) and that came closest to killing 50% of the larvae (equating to 5 × 10^7 per ml) within five days. For the experiments in the current study, all larvae were deprived of food for 2 h prior to commencing experimental feeding, and divided into three groups of 30 fourth instar larvae, to receive (i) sublethal, (ii) half-lethal or (iii) control treatments. The larvae were then maintained in Petri dishes with continuous access to artificial diet containing B. thuringiensis (1 ml of sublethal or half-lethal spore–crystal mixture per 3 g of diet), or artificial diet containing sterile water (1 ml per 3 g of diet) in the case of the control insects. The mortality of insects was 0.9%, 12.3% and 14.9% on the first, second and third day respectively following sublethal infection of insects with Bt. By the fifth day, total mortality was 17.1%. The half-lethal concentration of Bt (LC50) resulted in mortality of insects on the first (14.6%), second (40.2%) and third (45.6%) days after the treatment. By the fifth day total mortality was 46.8%. After 24 h, 48 h and 72 h exposure, surviving larvae were randomly selected to estimate the cellular and humoral immunity for control (diet with sterile water), sublethal (diet with bacteria (LC15)) and half-lethal (diet with bacteria (LC50)) treatments. Experiments were carried out triplicate, and separate groups of larvae were used to assay each separate immune response. The total numbers of larvae examined for each time interval per treatment were: for phagocytic activity of haemocytes (n = 30 larvae), encapsulation response (n = 40–80 larvae), phenoloxidase (PO) activity in haemocytes (n = 30 larvae), total haemocyte count (n = 10 larvae), PO activity of the haemolymph (n = 30 larvae), lysozyme-like activity of the haemolymph (n = 40 larvae), and coagulation (n = 25 larvae).

2.3. Phagocytic activity of haemocytes

The phagocytic activity of haemocytes was assessed using FITC-labelled Escherichia coli as previously described (Dubovskyi et al., 2008), with some modifications. The fluorescently labelled bacteria were injected into the insect haemocoel (10^6 cells in a volume of 5 μl per larva), and the phagocytic activity of haemocytes was investigated 1 h post injection. Phagocytosed fluorescent bacteria were visualised by fluorescence phase-contrast microscopy (Axioskope 40), and phagocytic activity was expressed as the percentage of haemocytes containing fluorescent E. coli (Rohloff et al., 1994).

2.4. Encapsulation response

The strength of the encapsulation response was measured as the degree of melanization of a “neutral antigen” nylon monofilament that was inserted into the insect haemocoel. This is a commonly used technique to gauge the general strength of insect immune responses (e.g. Kryukova et al., 2011; Rantala and Roff, 2006) and the cellular encapsulation response (Dubovskyi et al., 2013).

Implants consisted of pieces of white nylon monofilament with a knotted end, each 2 mm long and 0.5 mm in diameter. A single implant was inserted into the haemocoel of each larva through perforation of the ventral segment in the cuticle. One larva was
used for each sample. Implants were dissected from the body cavity after 2 h of exposure and then photographed from three angles. The degree of the melanization was quantified using Image Pro software by first measuring the colouration (grey value) of all areas of each implant, and then comparing these values with that of an intact implant (without melanization) (Dubovskiy et al., 2013).

2.5. Haemocytic phenoloxidase activity

A 10 µl suspension of haemocytes, obtained as described above (Section 2.3), was placed on a glass slide for 15 min in a moist chamber to form a monolayer, and then fixed with cold acetone for 10 min and washed thrice in distilled water. The haemocytes were then washed three times in phosphate buffer (PBS; 10 mM, pH 7.2) and incubated with 100 µl of i-DOPA (4 mg/ml) for 40 min in the dark at 28 °C. The monolayer was washed three times with distilled water and then observed by light microscopy (Zeiss; Axioskop 40). Cells with a dark brown colour were counted as PO-positive haemocytes, and haemocytic PO activity was expressed as the percentage of haemocytes with PO activity (Kryukova et al., 2011).

2.6. Total haemocyte count

A 5 µl sample of fresh haemolymph per larva was diluted 3-fold with ice cold AC containing PTU (4 mg/ml) to suppress melanization. Total haemocyte counts (THC) were immediately performed using a haemocytometer and were expressed as the number of haemocytes per ml of undiluted haemolymph. One larva was used for each sample.

2.7. Phenoloxidase activity in the haemolymph plasma

The phenoloxidase (PO) activity assay method was modified from Ashida and Soderhall (1984). 5 µl of haemolymph from each larva were collected into 20 µl ice-cold PBS containing the protease inhibitor PMSF (1 mM). The solution was centrifuged at 500g, 4 °C, for 5 min and 15 µl of the cell-free supernatant were added to microtitre plate wells containing 250 µl of 10 mM i-DOPA as a substrate for melanization reactions. One larva was used for each sample. After incubation at 28 °C for 30 min in the dark, the absorbance of the samples was measured at 490 nm using a Multiskan Ascent plate reader (Thermo). PO activity was expressed in units of transmission density (AA) of the incubation mixture per 1 min reaction time, per mg of protein.

2.8. Lysozyme-like activity of plasma

Antibacterial activity of haemolymph plasma was determined by an inhibition zone assay as described by Wojda et al. (2004). 10 µl aliquots of haemolymph from each larva were collected into 2 µl ice-cold PBS containing PTU (4 mg/ml). The solution was centrifuged for 5 min at 500g and 4 °C. Lysozyme-like activity in the resulting haemocyte-free plasma was detected using a radial-diffusion assay on 1.5% agarose plates at physiological osmolarity (NaCl 0.9%) containing 4 mg/ml freeze-dried M. lysodeikticus as substrate. Holes of 1 mm diameter were cut into the agar, into which 5 µl of sample was introduced and incubated at 28 °C for 18 h. One larva was used for each sample. Lysozyme-like activity was measured as a zone of digested M. lysodeikticus peptidoglycan, quantified using a standard curve made with EWL according to Mohrig and Messner (1968) and expressed as an EWL equivalent (mg/ml).

2.9. Coagulation assay

Haemolymph coagulation in response to an immune elicitor was assessed by measuring the dilution of a dye (Amaranth red) co-injected with LPS into the insect haemocoel (Haine et al., 2007). Any dye that becomes trapped in the haemolymph coagulum cannot subsequently diffuse into the haemocoel, and therefore the concentration of circulating dye is more dilute. A 2% solution of Amaranth red (Sigma) was made up in Insect Ringer solution (128 mM NaCl, 18 mM CaCl2, 1.3 mM KCl, 2.3 mM NaHCO3), and a second solution was made up in Insect Ringer supplemented with LPS (0.5 mg/ml). G. mellonella larvae were injected with 5 µl Amaranth dissolved in LPS solution in the posterior larval segments. Thirty min after injection a 5 µl sample of haemolymph was taken from an anterior part of the larvae. To prepare the standard curves for LPS/PBS dilutions, 5 µl of Amaranth solution (2% Amaranth red in PBS containing LPS at 0.5 mg/ml) was diluted in 25, 50, 100, 200, 300 and 400 µl in PBS, respectively. 5 µl of each haemolymph or standard sample was added to 195 µl PBS/LPS solution in a 96-well plate and the units of transmission density (AA) at 520 nm were measured on a Multiskan Ascent plate reader (Thermo). The mean AA 520 nm reading for each individual was converted to dye dilution factor in insect haemolymph, based on the standard curves.

2.10. Protein concentration

The protein concentration of haemolymph was estimated by the method of Bradford (1976), using bovine serum albumin (BSA) standards.

2.11. Statistical analyses

Data are presented as mean ± the standard error. To check the data for normal distribution, the Wilk Shapiro W criterion was used. The effect of bacterial infection on phagocytic activity of haemocytes, the encapsulation response, PO activity in haemocytes, total haemocyte count, PO activity of the haemolymph plasma, and coagulation, was assessed using one-way ANOVA, followed by Tukey’s post-hoc tests to identify specific differences between means. Lysozyme-like activity of the haemolymph analysed using the non-parametric Mann–Whitney U-test (STATISTICA 6.0).

3. Results

3.1. Phagocytic activity of haemocytes

Control insects had an average 26.63% of phagocytosing haemocytes (±0.86% over the experiment). Significantly increased
haemocyte phagocytic activity was detected in larvae infected with the sublethal concentration (LC15) of Bt, compared with the control (uninfected) larvae on the second \( (F = 3.02; \text{df} = 17; p = 0.045) \) and particularly the third \( (F = 8.26; \text{df} = 18; p \leq 0.001) \) days after the treatment (Fig. 1). The half-lethal concentration of Bt (LC50) reduced the phagocytic activity of haemocytes of G. mellonella larvae 2-fold on the third \( (F = 3.72; \text{df} = 18; p = 0.044) \) day of the infection (Fig. 1).

3.2. Encapsulation response

The melanotic encapsulation of nylon implants was enhanced in larvae infected with the sublethal Bt concentration, compared with the control larvae on the second \( (F = 1.72; \text{df} = 83; p = 0.034) \) and third \( (F = 1.16; \text{df} = 89; p = 0.007) \) days after the treatment (Fig. 2). However, G. mellonella larvae infected with the half-lethal concentration of Bt had a diminished encapsulation response to nylon implants compared with the controls on the second day \( (F = 1.12; \text{df} = 80; p = 0.004) \) and third day \( (F = 2.52; \text{df} = 74; p = 0.007) \) (Fig. 2).

3.3. Phenoloxidase activity in haemocytes

Control insects had an average 9.73% of haemocytes exhibiting phenoloxidase activity (±0.43% over the experiment). No significant differences were detected in the percentage of phenoloxidase-active haemocytes, between the larvae infected with the sublethal Bt dose and the control larvae (Fig. 3). However, for the larvae infected with the half-lethal Bt dose, a significant 1.4 fold reduction haemocytic phenoloxidase activity was detected when compared with control larvae during all the days of the experiment \( (F = 1.14; \text{df} = 16; p = 0.002) \) \( (F = 6.97; \text{df} = 17; p = 0.018) \) \( (F = 1.18; \text{df} = 15; p = 0.001) \) (Fig. 3). Significant differences in haemocyte PO activity were detected between sublethal infected larvae and larvae exposed to the half-lethal Bt dose on the first \( (F = 2.17; \text{df} = 16; p = 0.006) \), second \( (F = 2.42; \text{df} = 15; p = 0.001) \) and third \( (F = 2.3; \text{df} = 17; p = 0.001) \) days after treatment, with PO activity being consistently lower with the half-lethal Bt dose (Fig. 3).

3.4. Total haemocyte count

Control insects had an average total haemocyte count (THC) of \( 2.7 \times 10^8 (±1.2 \times 10^8) \) over the experiment. The sublethal Bt exposure significantly reduced the THC in infected larvae compared with the controls on the third day after treatment \( (F = 1.54; \text{df} = 15; p = 0.001) \) (Fig. 4). The THC decreased significantly on the second \( (F = 2.46; \text{df} = 15; p = 0.045) \) and third \( (F = 2.04; \text{df} = 14; p = 0.016) \) days after the half-lethal infection (Fig. 4).

3.5. Phenoloxidase activity in haemolymph plasma

A 1.2–1.5 fold increase of PO activity in the plasma was detected following peroral treatment of G. mellonella with the sublethal concentration of Bt on the first \( (F = 12.78; \text{df} = 53; p = 0.001) \), second \( (F = 1.11; \text{df} = 49; p = 0.001) \) and third \( (F = 1.55; \text{df} = 39; p = 0.001) \) days of the experiment (Fig. 5). The half-lethal concentration of Bt also induced higher PO activity in the plasma of infected insects on the first \( (F = 1.06; \text{df} = 54; p = 0.037) \), second \( (F = 1.91; \text{df} = 47; p = 0.042) \) and third \( (F = 1.13; \text{df} = 32; p = 0.023) \) days after the treatment compared with the controls (Fig. 5). Plasma PO was consistently higher in sublethal infected larvae.
compared with larvae exposed to the half-lethal concentration of Bt, and this was significant on the first \((F = 13.5; \ df = 55; \ p = 0.016)\) and third \((F = 1.75; \ df = 41; \ p = 0.001)\) days of the experiment (Fig. 5).

3.6. Lysozyme-like activity of the haemolymph plasma

The sublethal bacterial infection caused a significant increase in the lysozyme-like activity of the plasma on the first \((n = 67; \ U = 368.5; \ z = 2.4; \ p = 0.016)\), second \((n = 77; \ U = 359; \ z = 3.88; \ p \leq 0.001)\) and third \((n = 38; \ U = 111; \ z = 2.02; \ p = 0.043)\) days after the treatment (Fig. 6). Similarly, elevated plasma lysozyme activity was detected after ingestion of the half-lethal concentration of Bt on the first \((n = 69; \ U = 338; \ z = 3.06; \ p = 0.002)\), second \((n = 78; \ U = 423; \ z = 3.37; \ p \leq 0.001)\) and third \((n = 34; \ U = 85; \ z = 2.12; \ p = 0.033)\) days of the experiment (Fig. 6).

3.7. Coagulation index

There was no significant difference in the coagulation index of the haemolymph between larvae infected with the sublethal Bt dose and uninfected controls (Fig. 7). However, at the half-lethal concentration of Bt, a decrease in coagulation activity in infected insects was observed at all three days after treatment \((F = 1.52; \ df = 36; \ p = 0.008)\) \((F = 1.25; \ df = 32; \ p \leq 0.001)\) \((F = 1.02; \ df = 34; \ p = 0.002)\) respectively (Fig. 7). It should also be noted that, during the whole experiment, larvae exposed to the half-lethal Bt dose exhibited a reduction in coagulation activity relative to the larvae receiving the sublethal Bt dose \((F = 1.56; \ df = 40; \ p = 0.048)\) \((F = 1.43; \ df = 31; \ p = 0.007)\) \((F = 1.26; \ df = 43; \ p = 0.039)\) (Fig. 7).

4. Discussion

Our results suggest that a naturally administered (oral) sublethal dose of Bt elevates at least two arms of the cellular immune response in G. mellonella larvae: phagocytosis and encapsulation. In our previous investigations, similar enhanced activities of cellular immune reactions have been observed two and three days after sublethal Bt infection (Dubovskiy et al., 2008). The data also corroborate findings of increased immune resistance in the Mediterranean flour moth Ephesia kuehniella to Bt. In E. kuehniella, resistance was induced by pre-exposure to a low Bt dose, and correlated with an elevated haemolymph melanization reaction (Rahman et al., 2004). It is probable that a sublethal Bt dose leads to minor, but repairable, damage of the midgut epithelium cells, sufficient to elicit immune reactions in the haemolymph but not enough for total midgut destruction. An enhanced immune response could be elicited by foreign agents such as Bt spores and microbial metalloproteinases or peptidoglycan fragments, (Altincieek et al., 2007), members of the gut microflora, or signaling molecules generated by the insect’s own damaged intestinal cells (Broderick et al., 2009). Such minor damage to the midgut tissue might be repaired by stem cell proliferation (Baton and Ranford-Cartwright, 2007; Buchon et al., 2010; Castagnola et al., 2011). However, enhanced immunity also may be the relevant factor in limiting sublethal bacterial infection in the gut.

Sustained, enhanced levels of plasma lysozyme-like antibacterial activity were recorded soon post peroral infection of G. mellonella with both Bt doses in the current study. Such activities are thought to play a key role in inactivating bacteria sequestered in melanotic clots (Eleftherianos and Revenis, 2011). Moreover a plethora of antibacterial peptides are upregulated in the haemolymph of cabbage looper (Trichoplusia ni) larvae following exposure to low doses of Bt ssp. kurstaki (Ericsson et al., 2009). Broderick et al. (2010) found that intestinal Bt infection in gypsy moth Lymantria dispar larvae resulted in Bt toxin-induced perforative damage to the gut, which rapidly lead to escape of the intestinal symbiotic microflora into the insect haemocoele causing septicemia. Thus, it seems that the antibacterial system can protect an organism both from intestinal symbiotic microflora and Bt. Although Bt obviously begins its infection as a gut-confined organism, the vegetative cells, spores and parts of intestinal cells can leak into the insect haemocoel through damaged midgut tissue (Griffitts and Aroian, 2005). In this case the contamination of haemolymph by components of the destroyed intestinal cells, bacterial and microflora cells, can lead an overwhelming or suppressing of the cellular immune reactions under high (half-lethal) doses of Bt. In our study, haemocytes were depleted more rapidly in the half-lethal infected insects, although by three days both treatments resulted in low THCs of similar concentrations. Other studies have reported significant decreases in the number of circulating haemocytes of gypsy moth L. dispar and cabbage looper T. ni larvae after per os challenge with Bt (Broderick et al., 2010; Ericsson et al., 2009). In addition THC losses have been reported following treatment of Agrotis ipsilon and Rhynchochoporus ferrugineus with both sublethal and half-lethal concentration of Bt (Abd El-Aziz and Awad, 2010; Manachini et al., 2011). It is known that the recruitment of G. mellonella haemocytes for cellular immune reactions can result in a depletion of their pool (Rowley and Ratcliffe, 1978). Moreover, insects infected by high concentrations of Bt exhibit symptoms such as cessation of feeding, weight loss and stunted development compared with control insects (Bravo et al., 2005). During short-term nutritional deprivation the activity of immune reactions is down-regulated (Siva-Jothy and Thompson, 2002). A decrease in the number of circulating haemocytes registered in our study under Bt infection was probably the
result of the cells participating in immune reactions and/or it may be linked with starvation of infected insects during intestinal Bt infection.

It is difficult to account for the enhanced cell-mediated immune activities in sublethal infected insects, especially on day 3 post infection. Possibly, subpopulations of haemocytes are affected differently by the two Bt doses. In the case of the half-lethal dose, haemocytes might be quickly and substantially recruited to sites of intestinal damage (e.g. to seal perforations and to attack leaking gut contents); melanization may be a key feature of such repair activities. Our experiments showed that both concentrations of Bt induce PO activity in haemolymph plasma of larvae, which is sustained for three days post infection. Moreover the sublethal Bt infected larvae exhibited higher PO activity in the plasma compared with the larvae infected with the half-lethal Bt dose. Rahman et al. (2004) also reported a 20-fold increase in plasma PO activity in the larvae of loopers T. ni after a sublethal Bt treatment. Melanotic spots have been detected on the gut peritrophic matrix of cotton bollworm Helicoverpa armigera larvae resistant to Bt toxin that also have elevated PO activity in cell-free haemolymph (plasma) and gut extracts (Ayres and Schneider, 2008; Ma et al., 2005). A recent study using isolated Morduca sexta PO indicates that two compounds produced by this enzyme, dopamine and 5,6-dihydroxyindole, exhibit a high antibacterial activity (in vitro) against several bacterial species (including Bacillus cereus and Bacillus subtilis) (Cerenius et al., 2008). It might be supposed that the immune components from the haemolymph, such as proPO, take part in inducible humoral defence reactions of wax moth during bacterial Bt infection. PO participates in melanization and repairs perforations in the midgut to help prevent bacteria escaping into the haemocoel by exuding antibacterial substances in the damaged tissue. Careful microscopic analyses of infected guts will be needed to explore this hypothesis, but it is supported by previous studies which show that chemical or biological suppression of the key immune reactions can both enhance the susceptibility of insects and reduce recruitment of the insect’s resistance to intestinal Bt infections (Broderick et al., 2010; Kwon and Kim, 2007; Richards and Dani, 2010).

It is interesting that in the current study, the half-lethal Bt dose resulted in decreased coagulation indices of larval haemolymph compared both with the control larvae and those larvae infected with the sublethal Bt dose. Very little published information exists regarding insect coagulation indices during bacterial intestinal Bt infections. It is known that some pro-coagulant glycoproteins can inactivate Bt toxin (Ma et al., 2012, 2005). In light of the current data, it would be interesting to determine whether suppressed coagulation correlates with lower haemolymph concentrations of known coagulation elicitors (such as lipophorin, Ca²⁺ or transglutaminase) (Altincicek et al., 2008; Dushay, 2009; Li et al., 2002) or haemocyte depletion required for protection against Bt.

In summary, our data reveal substantial stimulation of the humoral and cellular immune responses of G. mellonella larvae during intestinal sublethal Bt infections. The nature and strength of the Bt-induced immunity was clearly dose-dependent, since higher Bt concentrations led to a marked reduction in key immune responses. The elevated activities probably assist the insect both in overcoming low-level infections and in avoiding septicaemia and death associated with gut perforation. While enhanced humoral defence reactions (lysozyme-like and PO activity) occur during acute intestinal Bt infection, at the higher concentration of Bt, depletion of the haemocyte pool and strong intoxication could lead to a trade-off between cellular and humoral immunity. Based on the current data we can suggest that both cellular and humoral immune reactions make important contributions to insect inducible resistance to Bt. Moreover this study highlights important dose-dependence effect of the bacterial inoculum that should be considered when designing biocontrol programmes.

Acknowledgments

We thank Dr. Natalia Kryukova for fruitful advice for the study and for producing haemocyte images. This study was supported by the Russian Foundation for Basic Research (14-04-3150) and a presidential grant.

References


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