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Low cost antiviral activity of *Plodia interpunctella* haemolymph *in vivo* demonstrated by dose dependent infection

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ABSTRACT

Given the ubiquity of infectious disease it is important to understand the way in which hosts defend themselves and any costs that they may pay for this defence. Despite this, we know relatively little about insect immune responses to viruses when compared to their well-characterized responses to other pathogens. In particular it is unclear whether there is significant haemocoelic response to viral infection. Here we directly examine this question by examining whether there is a dose-dependency in infection risk when a DNA virus is injected directly into the haemocoel. Infection from direct injection into the haemocoel showed a clear dose dependency that is indicative of an active intrahaemocoelic immune response to DNA viruses in insects. In contrast to the natural oral infection route, we found no measurable sublethal effects in the survivours from direct injection. This suggests that the immune responses in the haemocoel are less costly than those that occur earlier.

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1. Introduction

There is an increasing interest in determining the mechanisms of insect immunity. This interest is driven by the insights that can be gained into the mammalian innate immune system (Lowenberger, 2001; Kanost et al., 2004) and the growing field of evolutionary immunology which emphasizes the importance of costs to using and maintaining immune systems (Schmid-Hempel, 2003; Miller et al., 2005; Rolff and Reynolds, 2009). These costs are important because they define the optimal level of resistance and may generate and maintain genetic diversity in hosts (Boots and Begon, 1993; Boots and Bowers, 1999; Boots and Haraguchi, 1999; Miller et al., 2006; Best et al., 2008). An understanding of the mechanisms of insect resistance to infection is also important to the control of vectorborne viral disease and the biological control of pest insects (Rolff and Reynolds, 2009). We now know a great deal about how insects resist bacterial, parasitoid and fungal infection, but we know much less about how insects resist viruses (Imler and Eleftherianos, 2009). Given the ubiquity of viruses in nature, and the fact that many important human viruses, such as dengue, are vectored by insects, there is a pressing need to understand how insects resist viral infection.

Baculoviruses are DNA viruses with two distinct forms, the occlusion virus and the budded virus, that complete the infection cycle within the insect host (Granados, 1983). The occlusion virus is the naturally infectious stage, and infects midgut cells after being ingested under alkaline conditions in the gut lumen. The budded viruses then enter the insect haemocoel and infect the secondary target cells (Granados, 1983). Because of their high pathogenicity and generally limited host range the baculoviruses have received a lot of attention as pest control agents (Moscardi, 1999). Baculoviruses have also been used as a model for both host parasite evolution (Boots and Mealor, 2007) and for the study of insect immune defence mechanisms against viral infection (Boots and Begon, 1993, 1994, 1995). The defence mechanisms that are known to operate are gut pH, midgut digestive juices, antiviral digestive enzymes (Nakazawa et al., 2004) and the peritrophic membrane (Granados, 1983; Wang and Granados, 1998). Sloughing of infected midgut cells has also been demonstrated as an effective mechanism against several pathogens, including virus, which enter the insect host by the oral route (Engelhard and Volkman, 1995). However we know very little about how insects respond to viruses once they pass through the midgut and enter the haemocoel. Indeed it is unclear whether there is a significant response to the budded virus in the haemocoel.

We directly examine the question of whether there is a response in the haemocoel by determining whether there is a dose-dependency in infection risk when a budded granulosis virus is

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injected into the haemocoel. This is compared with the dose dependency in response to the natural oral transmission route. We examine whether there are any costs of using the haemocoelic resistance by examine sublethal effects on the development time and size of survivours. Sublethal effects to the natural oral infection of baculoviruses are well established and may have important population dynamical effects (Boots and Begon, 1994; Sait et al., 1994; Rothman and Myers, 1996; Boots and Norman, 2000; Boots et al., 2003). Direct injection of virus into the insect's haemocoel is interesting because it bypasses these primary defences and we can therefore examine whether there are sublethal effects to later immune responses. The standard method used for obtaining budded NPVs virus for injection, is via, cell culture. Although a great deal of useful information can be obtained from *in vivo* infection, a complete investigation of the infection process requires *in vitro* manipulation of the virus and cells under controlled conditions. In addition, granulosis viruses appear not to infect cells *in vitro*, and no suitable tissue culture system has yet been found to support their replication. We therefore first develop an *in vivo* technique to produce budded granulosis virus; the form that infects within the haemocoel. Our results show a clear dose dependency to intrahaemocoelic infection that reflects an active immune response to DNA viruses in the haemocoel, but that the costs of using this system are much lower than to natural infection.

2. Methods

2.1. Preparation of occlusion virus

We used the Indian meal moth, *Plodia interpunctella*, and its granulosis virus (PiGV) as our model system and examined the dose dependency of infection from the natural oral infection route and via direct injection. The natural occlusion virus is routinely amplified in *P. interpunctella* larvae and extracted by sucrose gradient centrifugation. Purified occlusion virus was stored at −20 °C in 250 μl aliquots, and each aliquot was used only once and subsequently discarded. The occlusion virus stock used for oral inoculation contained 6.9×10^7 viral particles per ml and serially diluted in a colored dosing fluid (1% sucrose, 0.1% Coomassie brilliant blue dye), to the following concentrations (1) 6.9×10^6, (2) 6.9×10^5, (3) 6.9×10^4, (4) 6.9×10^3, (5) 6.9×10^2, (6) 69 and (7) 6.9 viral particle per ml.

2.2. Oral innoculation

We used a modified droplet feeding technique for inoculation (Hughes and Wood, 1981), with 50 larvae at each virus concentration. Fourth instar larvae were starved in a petridish diameter 9 cm for 1 h prior to oral feeding with virus. Small droplets of the diluted virus solution (approximately 0.5 μl in size) were then placed in each Petridish, placed in an incubator for 2 h, and then thirty larvae which had ingested half a gut's length or more of virus suspension (judged by eye) were placed individually in 7 ml clear plastic containers with 2–3 small holes on the lid, and fed with fresh artificial diet. The larvae were maintained at 27 ± 2 °C, 70 ± 5% on an 16 h light:8 h dark regime.

2.3. Preparation of budded virus

Budded virus was harvested from the haemolymph of larvae that had been orally infected as described above early in the 4th instar (14 days old). In preliminary experiments, we examined the effect of different times of harvesting the virus and found that none of recipient larvae developed disease symptoms if the haemolymph was harvested within 5 days of oral innoculation. Haemolymph that was therefore harvested after 6–8 days, which gave a combination of a high level of infection with minimal larva mortality. The budded virus used for direct intrahaemocoelic injection in the experiments was therefore extracted from the haemolymph of orally challenged larvae after 7 days. A technique was developed to quantify the amount of haemolymph taken from each of the donor larva, using a calibrated glass needle. Immobilised donor larvae were placed under a stereo-microscope and covered with cling film (to increase the pressure on the larva). The tip of the proleg was pricked using a small needle, and haemolymph was collected with a calibrated glass needle by capillary action. The collected haemolymph was expelled into 50 μl of sterile water in a 500 μl vial. Haemolymph from all donor larvae was collected and the volume of haemolymph for each larva was recorded. The haemolymph was gently mixed and serial diluted with sterile distilled water.

2.4. Intrahaemocoelic injection

The actual number of budded virus particles in the haemolymph was not quantified because the survival of the budded virus is poor (we found that no infection occurs if the budded virus is kept for more than 24 h). Therefore, the viral concentration used for analysis was the concentration of haemolymph in the sterile distilled water. We made seven serial dilutions: (1) 3×10^{-1}, (2) 3×10^{-2}, (3) 3×10^{-3}, (4) 3×10^{-4}, (5) 3×10^{-5}, (6) 3×10^{-6} and (7) 3×10^{-7}. As a control haemolymph was harvested from non-challenged individuals used at the highest concentration (3×10^{-1}). Thirty larvae were used at each virus concentration with another thirty for the controls. As a further control, 30 larvae were injected with sterile water. All injected larvae were placed individually in 7 ml clear plastic containers with 2–3 small holes on the lid, and fed with fresh artificial diet. The larvae were maintained at 27 ± 2 °C, 70 ± 5% on a 16 h light:8 h dark regime.

2.5. Infection rates, sublethal effects and analysis

The experimental larvae were observed daily, and examined for disease symptoms: infected larvae are easily distinguished by their opaque white color (Vail and Tebbets, 1990). In order to obtain a high level of replication, the experiment was carried out in 5 blocks over a three-month period. In one of the blocks, we in addition, examined whether there were sublethal effects on developmental time and size by recording the time to pupation and pupal weight of the individuals that did not become infected. The male larvae were distinguished from female larvae by the presence of testes, which are visible through their dorsal surface. Mortality was negligible throughout the experiment and showed no variation between the treatments. Statistical analysis was carried out using the statistical package R (version 2.2). Dose mortality data were analysed using a general linear model with binomial errors. The sublethal effects of both oral and intrahaemocoelic injection on pupal weight and development time were examined by using analyses of variance (ANOVA), where the explanatory factors were larval sex and treatment. Dose-dependent sublethal effects were not tested because the number of surviving larvae from each viral dose was too small, particularly at high viral doses. All the dilutions were therefore combined leading to five larval treatment groups: control non-challenged larvae, larvae injected with sterile water, larvae injected with non-infectious haemolymph, larvae injected with budded virus and orally inoculated larvae. We decided *a priori* to carry out contrasts that compared each of the treatment groups in turn with the controls. The dose response of each larval treatment group was analysed using a logistic analysis of deviance to compare the response to the viral dose used in the oral inoculation between larval treatment groups.

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