Baculovirus replication alters hormone-regulated host development

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The baculovirus Lymantria dispar nuclear polyhedrosis virus interferes with insect larval development by altering the host’s hormonal system. The level of haemolymph ecdysteroids, the insect moulting hormone, was found to be higher in virus-infected larvae than in uninfected controls. This was consistently observed in both fourth instars and day 5-infected fifth instars. The rate of hormone synthesis was examined by in vitro incubation of the prothoracic gland. Gland activity in virus-infected larvae was higher than controls and continued until the late stages of virus infection, even during the time that controls had ceased to secrete ecdysone after moulting. During virus replication, the prothoracic gland was observed to maintain morphological and ultrastructural characteristics indicative of ecdysone biosynthetic activities. Therefore, it is likely that the insects are no longer under the control of the normal hormonal system after virus infection. It is felt that the alteration of hormone titre and the rate of ecdysone synthesis is the result of the activity of ecdysteroid UDP-glucosyl transferase (EGT), a virus-encoded enzyme which is thought to inactivate ecdysteroids by sugar conjugation.

Introduction

Baculoviruses are the most prominent group of viruses infecting lepidopteran insects. Infection generally begins with ingestion of virus-contaminated food and is poly-organotropic, involving numerous tissues including midgut cells, trachea, epidermis and fat body (Granados & Williams, 1986; Volkman & Keddie, 1990). It has been reported that, during virus replication, infected insects fail to moult (O’Reilly & Miller, 1989; Burand & Park, 1992). This effect has been attributed to the activity of a virus-encoded enzyme, an ecdysteroid UDP-glucosyl transferase (EGT; O’Reilly & Miller, 1989). Alternatively, it has been proposed that this inhibition of moulting is due to infection of the prothoracic gland, the tissue that produces the moulting hormone (Volkman & Keddie, 1990).

The baculovirus egt gene encodes the enzyme EGT which catalyses the transfer of a sugar moiety from UDP-sugars to ecdysteroids (O’Reilly & Miller, 1989). Ecdysteroid is traditionally viewed as the moulting hormone in insects and it is well established that it functions as the hormone controlling development and reproduction (Smith, 1985; Lafont et al., 1986). Since the first discovery of the egt gene in the baculovirus Autographa californica multicapsid nuclear polyhedrosis virus (AcMNPV), a significant amount of information has been obtained on the molecular biology and biochemistry of EGT (O’Reilly & Miller, 1989, 1990; O’Reilly et al., 1991). O’Reilly et al. (1992) reported that egt gene expression enables the virus to manipulate insect larval development by sugar conjugation of ecdysteroids and this conjugate has been reported to be an ecdysone galactoside in AcMNPV-infected Spodoptera frugiperda larvae.

In our previous study, we reported that Lymantria dispar nuclear polyhedrosis virus (LdNPV) infection affects host (gypsy moth) larval development by slowing weight gain and inhibiting larval moulting (Burand & Park, 1992). We have also demonstrated that LdNPV-infected cells produce EGT in vitro and virus infection affects the level of haemolymph ecdysteroids in the host insect, possibly through the expression of the egt gene (Park et al., 1993).

Generally, ecdysteroids present in insect haemolymph fluctuate systematically with respect to both concentration and time (Smith, 1985). The titre of the hormone is strictly regulated at characteristic levels during insect post-embryonic development and the fluctuation in titre is correlated with the developmental stage and physiological state of the insects. We have found that in virus-infected larvae, however, both the level and pattern of the haemolymph ecdysteroids are different from those in uninfected control insects. Furthermore, the response was different depending on the stage of the insect. Virus-infected fourth instars showed very high ecdysteroid

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levels while fifth instar larvae had very low levels of ecdysteroids in the haemolymph when they were infected at day 1 (Park et al., 1993). In the present study, we have investigated the reason for the differences in the responses in the two processes by changing the time of infection of fifth instars. Throughout the study with fifth instar larvae infected at day 5, we report consistently high levels of ecdysteroids in baculovirus LdNPV-infected L. dispar larval haemolymph.

Based on the data from several classical studies (Chino et al., 1974; King et al., 1974; Bollenbacher et al., 1979), the prothoracic glands are accepted as the primary physiological source of ecdysone during most of post-embryonic development. Considering the high levels of ecdysteroids in virus-infected larvae we have conducted research on the prothoracic glands to investigate their activity during virus infection. In this paper we report a significant influence of virus replication on insect hormonal synthesis by the prothoracic glands.

The relationship between glandular structure and function has been investigated in several insect systems (Herman, 1967; Sedlak et al., 1983; Hanton et al., 1993). Changes in the prothoracic gland ultrastructure during larval–larval and larval–pupal development have been observed and these structural dynamics are believed to be closely related to the activity of the glands. Since we had found an increase of hormone in the haemolymph and altered activity of the prothoracic glands during virus replication we began to investigate the effect of baculovirus infection on the morphology and ultrastructure of gland cells. In this study, we observed that the morphology and ultrastructure of the glands correlated with the alteration of the hormonal system during virus replication.

Methods

Animals and viruses. Egg masses from the New Jersey strain of the gypsy moth (L. dispar) were obtained from the US Department of Agriculture (Otis Methods Development Center, Otis ANGB, Mass., USA) and larvae hatched from eggs were reared on an artificial wheatgerm diet at 27 °C (Bell et al., 1981). The wild-type LdNPV isolate Gyplchek was propagated in insects and virus occlusion bodies (OBs) were purified from infected larval cadavers. For infection, newly ecdysed fourth instar female larvae (day 1) and fifth instar female larvae at five days after ecdysis (designated as day 5) were selected and infected as described previously (Park et al., 1993).

Haemolymph ecdysteroid titre determination. Haemolymph was collected from each group of day 5-infected fifth instar larva at 24 h intervals starting at 1 day post-infection (d.p.i.). A 10 μl sample of haemolymph was collected from a pierced proleg and diluted into 90 μl of cold methanol. Samples were vortexed, centrifuged at 10000 g for 10 min and the supernatants were collected, vacuum-dried, resuspended in 80% methanol and stored at −20 °C until used. The amount of ecdysteroid in each sample was then measured by radioimmunoassay (RIA) using anti-ecdysone antiserum provided by W. Bollenbacher as outlined (Bollenbacher et al., 1975). The radiolabelled antigen was [3H]ecdysone (New England Nuclear) and ecdysone (2–2000 pg) was used to construct the standard curve.

In vitro incubation of prothoracic glands. At various times after infection prothoracic glands were dissected under lepidopteran saline (Weevers, 1966) and rinsed for 15 min in saline to remove contaminating haemolymph ecdysteroids. Individual glands were incubated in 25 μl of Grace’s medium (Gibco) for 12 h at 25 °C under a high humidity atmosphere (Bollenbacher et al., 1979; Kelly et al., 1992). After incubation, the culture medium was dried and re-extracted in 100 μl of 80% methanol. The ecdysone content was measured by RIA as described above.

Light microscopy. Prothoracic glands were removed from both uninfected control and virus-infected fourth instar larvae at 3.5 d.p.i. and 6 d.p.i. to observe the morphology. These times were chosen since 3.5 d.p.i. was found to be the time of peak of ecdysone secretion and 6 d.p.i. was the time at which controls moulted into fifth instars and had basal secretary activity. After dissecting under saline, the glands were immediately fixed with 2.5% glutaraldehyde in 0.1 M-sodium cacodylate buffer pH 7.2 for 4 h, washed twice in 0.1 M-sodium cacodylate buffer pH 7.2 and observed using a dissecting microscope.

Electron microscopy. For ultrastructural studies, glands were prepared and prefixed as described above then post-fixed with 1% osmium tetroxide in 0.1 M-sodium cacodylate buffer for 3 h at 4 °C in the dark, dehydrated and embedded in Spurr medium (Spurr, 1969). Thin sections of 60–100 nm were stained with uranyl acetate and lead citrate and then observed with a Philips CM 10 transmission electron microscope.

Results

Effect of virus infection on haemolymph ecdysteroid titre

The level of haemolymph ecdysteroids present in day 5-infected fifth instar larvae was observed and compared with uninfected controls at the same time (Fig. 1). The pattern of ecdysteroid titres in uninfected control fifth

![Fig. 1. Haemolymph ecdysteroid titre during larval-pupal moulting when insects were infected with LdNPV as day 5 larvae. Day 5 fifth instar larvae were infected with virus OBs (8 × 10⁶ OBs/insect). RIA was used to determine the concentration of ecdysteroid in haemolymph from control and virus-infected larvae. Each point represents the mean ± 1 SEM from at least five separate determinations.](image-url)
instar gypsy moth larvae was similar to that reported for other insects (Steel & Vafopoulou, 1989), showing a peak at the time of prepupae. In these larvae, the concentration of ecdysteroids remained at a low level until they became prepupae. The titre increased to a peak of 1080 pg/ml at 9 days after ecdysis to the fifth instar, which corresponds to 4 d.p.i. in this experiment, and dropped back to a base level in old prepupae. In contrast to the ecdysteroid titre in larvae infected at day 1 (Park et al., 1993), which showed basal levels without a peak, the level of ecdysteroids in day 5-infected insects was high during the time that control insects showed the peak in titre. Compared to controls, day 5-infected insects showed a higher ecdysteroid titre. The titre began to rise earlier (the virus-infected insects already showed more than 1000 pg/ml at 3 d.p.i.), had a higher peak titre and stayed at a high level longer than in the controls. Glands from virus-infected larvae contained greater amounts of ecdysteroid even after the time the control larvae ceased to secrete a detectable amount of hormone.

The same effect of virus infection was observed for glands from fifth instar larvae infected at day 5 (Fig. 3). The prothoracic glands from virus-infected larvae showed an increased secretion of ecdysteroids earlier than controls and maximum secretion at a higher level, while glands from control fifth instars showed a pattern similar to that found in control glands of fourth instars, with a peak (30 ng/gland) at the time of prepupae. Glands from virus-infected larvae began to secrete a detectable amount of ecdysteroid starting at 2 days after the ecdysis to the fourth instar and reached a peak at the time of apolysis, the initial signal of moulting, which corresponds to 3-5 d.p.i. Each prothoracic gland at this peak of ecdysteroid synthesis produced approximately 17 ng of ecdysone equivalents in vitro. The average titre of ecdysteroids produced by glands from virus-infected larvae was elevated concomitant with the increase in the haemolymph ecdysteroid titre and remained higher than in the controls. Glands from virus-infected larvae continued to produce large amounts of ecdysteroid even after the time the control larvae ceased to secrete a detectable amount of hormone.

Morphology of the prothoracic glands

Morphological changes of the prothoracic glands from control and virus-infected larvae were observed microscopically throughout the fourth larval stadium of development. Glands from several different insects were

Ecdysteroid synthesis by the prothoracic glands

The concentration of ecdysteroid secreted by the prothoracic glands from both uninfected control and virus-infected fourth instar larvae, which were infected at day 1, was measured by RIA (Fig. 2). The uninfected control
observed at different times after infection and the appearance of typical glands at 3.5 d.p.i. and at 6 d.p.i. is shown in Fig. 5.

The cells from each gland formed a distinct 'Y' shape and right and left glands had a similar morphology. At 3.5 d.p.i., each pair of glands from both control and virus-infected insects was found to be a compact tissue composed of large cells. No significant differences were observed in the morphology of glands from control and virus-infected larvae at this time.

At 6 d.p.i., the uninfected control insects had already moulted to the fifth instars and had smaller glands consisting of smaller cells compared to the active glands at 3.5 d.p.i. In contrast, glands from virus-infected insects were very large at this time, with a visible increase in the diameter of each cell even though these insects stayed as fourth instars without gaining a significant amount of weight (Burand & Park, 1992).

**Ultrastructure of the prothoracic glands**

Using electron microscopy, gland cells were found to be tightly joined to one another with some tracheolar cells associated with the gland. The most distinct features of individual cells were observed in the cell periphery. In active glands at 3.5 d.p.i., deep channels were observed on the cell surface, which are thought to be formed by plasma membrane invagination (Fig. 6a, b). Membrane infolding was very intense and correlated with the high levels of secretion of the hormone. The pattern of this channel system in cells of glands from both control and virus-infected larvae was similar at this time.

At 6 d.p.i., control insects, which had already moulted to the fifth instars, had a relatively narrow peripheral band as seen in Fig. 6(c). The cells in these glands contained large, irregular-shaped nuclei surrounded by a thin cytoplasm. The plasma membrane was relatively smooth with few infoldings and the cell surface area was covered with a thicker basal lamina compared to cells from glands at 3.5 d.p.i. Correlating with the reduced size of the cytoplasmic area, the numbers of organelles appeared to be significantly reduced. However, at the same time, the virus-infected larval prothoracic gland cells showed an extensive peripheral channel system (Fig. 6d). As mentioned above, the size of cells was large, mainly due to the increase in cytoplasmic area. Cytoplasmic organelles were also quite numerous. These insects stayed as fourth instar without moulting into the fifth and the basal laminar appeared as a narrow band covering the gland cells.

No virus OBs were observed in the prothoracic gland cells even though the glands were removed at 6 d.p.i. (Fig. 7a). Among the observed glands, no sign of virus replication was observed in any glandular cells. However, all the attached tracheolar cells in these samples were highly infected and contained virions, and virus OBs were observed in the nucleus (Fig. 7b). This observation was also true for gland cells from fifth instars at 8 d.p.i. (data not shown), which were infected at day 1. No virus particles were observed in the prothoracic gland cells, while severe degeneration of insect tissues with light blue-coloured, milky haemolymph was observed.

**Discussion**

The present study demonstrates that the baculovirus LdNPV manipulates host larval development by altering hormonal balance. Both fourth and fifth instar larvae showed a consistent pattern with high ecdysteroid levels in the haemolymph after virus infection. This study clearly demonstrates that LdNPV infection causes the alteration in the insect hormonal system, resulting in an increase in the hormone concentration. Since virus-encoded EGT is present in the haemolymph as a stable enzyme from early in infection, most of the ecdysteroids in virus-infected larvae are considered to be present as sugar-conjugated ecdysone, the product of EGT. This conjugated ecdysone is believed to be an inactive form of the moulting hormone, which means that the insects cannot moult even with a high level of ecdysteroids in the haemolymph. The antiserum used here to measure the concentration of ecdysteroids was found to recognize both ecdysone and the glucose conjugate (Park et al., 1993).

Since the synthesis of ecdysteroids appears to be a feature of fundamental importance contributing to
Baculovirus-modified host ecdysone synthesis

Fig. 5. Morphology of the prothoracic glands from control and LdNPV-infected gypsy moth larvae during fourth and fifth instar larval development. (a) Gland from uninfected control insect at 3.5 d.p.i.; (b) gland from virus-infected larva at 3.5 d.p.i.; (c) gland from uninfected control insect at 6 d.p.i.; (d) gland from virus-infected insect at 6 d.p.i. Bar marker represents 400 μm.

haemolymph ecdysteroid titre, the level of hormone synthesized by the prothoracic glands was investigated. In vitro culture of the prothoracic glands revealed that the glands from virus-infected larvae produced a higher level of ecdysteroid than control glands and continued to produce a considerable amount of hormone even after the time controls ceased to secrete detectable levels of ecdysteroid. These effects were true for both fourth instar (Fig. 2) and fifth instar larvae infected at day 5 (Fig. 3). The relationship between in vitro synthesis of hormone by prothoracic glands and the amount of circulating ecdysteroids was close and the profile of the haemolymph ecdysteroid titre was reflected by that of the gland activity. Therefore, the main reason for the high titre of ecdysteroids in virus-infected larval haemolymph is thought to be the high level of ecdysteroid synthesized by the prothoracic glands of these insects.

The morphology and ultrastructure of the gland and gland cells strongly support the continuous synthesis of ecdysteroids by the prothoracic glands in virus-infected larvae (Figs 5 and 6). The glands from virus-infected larvae remained large and were composed of large cells, even during the time when controls had moulted and had much smaller glands. In addition, an increased surface area of cell periphery, which generally occurs concurrent with the peak in ecdysone secretory activity, was observed in gland cells from virus-infected insects at 6 d.p.i. The appearance of distinct and prominent membrane invaginations in the cells of virus-infected insects at this stage of development strongly suggests the continuous activity of the prothoracic glands.

The activity of the prothoracic gland in the fifth instars infected at day 1 was also observed (Fig. 4). As expected from their haemolymph ecdysteroid titre, these glands produced no detectable ecdysteroids. Therefore, the low level of ecdysteroid which we had previously found in the haemolymph of these insects (Park et al., 1993) is due primarily to the lack of synthesis by the prothoracic glands. O'Reilly et al. (1992) also reported low levels of ecdysteroids in AcMNPV-infected insects. It appears that infection of insect tissues at an early stage of the fifth instar prevents activation of the prothoracic glands.
Fig. 6. Ultrastructure of prothoracic gland cells from control and virus-infected gypsy moth larvae during fourth to fifth instar larval development. (a) Gland from uninfected control insect at 3.5 d.p.i.; (b) gland from virus-infected insect at 3.5 d.p.i.; (c) gland from uninfected control insect at 6 d.p.i.; (d) gland from virus-infected insect at 6 d.p.i. Bar represents 1 μm. ICS, intercellular spaces; BL, basal lamina; NM, nuclear membrane; PM, plasma membrane; T, tracheole; m, mitochondrion.

Since no virus particles have been observed in the gland cells, it is likely that the inactivity of the prothoracic glands in these insects is not simply due to infection of the gland cells but possibly to a block in stimulation from the brain (or other tissues) caused by virus infection.

It has been postulated that the inhibition of moulting is due to replication of the virus in gland cells (Volkman & Keddie, 1990). In fact, virus replication has been reported in the prothoracic gland of other baculovirus-infected insects (Dougherty et al., 1987). Interestingly, no signs of virus replication were observed in any of the prothoracic glands recovered from LdNPV-infected gypsy moth larvae in this study. However, severe degeneration of other insect tissues such as tracheoles and fat body was observed both at 6 d.p.i. in fourth instars and at 8 d.p.i. in fifth instars. Therefore, the inhibition of moulting in gypsy moth larvae infected with LdNPV seems to be due to the activity of a virus-encoded gene product such as EGT and not a result of virus infection of the prothoracic gland.

These results provide the first demonstration of the continuous activity of the prothoracic glands in virus-infected insects. Although there is one report of ecdysone secretion from the prothoracic gland of granulosis virus-infected Trichoplusia ni larvae (Dougherty et al., 1987), the level of synthesis was lower than controls and the level of haemolymph ecdysteroids remained low in these insects.

Recently, O’Reilly et al. (1992) reported that the ecdysteroid titre in AcMNPV-infected S. frugiperda larval haemolymph stayed at basal level throughout virus replication. They suggested that the steady-state levels of ecdysteroids in the haemolymph remain low and that the ecdysteroids were cleared from haemolymph rapidly after EGT-catalysed conjugation. The results presented here, however, demonstrate that the baculovirus LdNPV modifies host ecdysteroid titre in a different manner. It appears that rapid clearing of the ecdysteroid conjugate suggested by O’Reilly et al. (1992) does not occur in the haemolymph of LdNPV-infected gypsy
Fig. 7. Virus infection of the prothoracic gland and tracheolar cells in LdNPV-infected gypsy moth larvae at 6 d.p.i. (a) Prothoracic gland cell; (b) tracheolar cell from virus-infected larva. NM, nuclear membrane; PM, plasma membrane; OB, occlusion body; v, virion.

moth larvae. The mechanism driving higher and continuous synthesis of the ecdysteroid in these insects is not yet clear, but could involve an alteration in the feedback regulation of ecdysteroid synthesis through the expression of the virus egt gene and the ecdysteroid-sugar conjugate produced by EGT. It is also possible that altered regulation by the brain or other tissues could be involved. Further research is needed to determine the mechanism involved in the activation of the prothoracic glands in virus-infected insects.

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