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Effect of starvation upon baculovirus replication in larval *Bombyx mori* and *Heliothis virescens*

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ABSTRACT

The progression of baculovirus (BmNPV, BmCysPD, AcMNPV or AcAaIT) infection in larval *Bombyx mori* and *Heliothis virescens* (1st, 3rd or 5th instar) was investigated following various starvation regimes. When the larvae were starved for 12 or 24 h immediately following inoculation, the median lethal time to death (LT_{50}) was delayed by 9.5–19.2 h in comparison to non-starved controls. This corresponded to a delay of 10–23% depending upon the larval stage and virus that was used for inoculation. When a 24 h-long starvation period was initiated at 1 or 2 days post inoculation (p.i.), a statistically significant difference in LT_{50} was not found indicating that the early stages of infection are more sensitive to the effects of starvation. Viral titers in the hemolymph of 5th instar *B. mori* that were starved for 24 h immediately following inoculation were 10-fold lower ($p < 0.01$) than that found in non-starved control larvae. Histochemical analyses indicated that virus transmission was reduced in 5th instar *B. mori* that were starved for 24 h immediately following inoculation in comparison to non-starved control larvae. In general, the mass of larvae that were starved immediately after inoculation was 30% lower than that of non-starved control insects. Our findings indicate that starvation of the larval host at the time of baculovirus exposure has a negative effect on the rate baculovirus transmission and pathogenesis.

1. Introduction

Baculoviruses are arthropod pathogenic viruses with circular, double-stranded DNA genomes and rod-shaped, enveloped virions (Bonning, 2005; Rohrmann, 2008). The family Baculoviridae is composed of two genera: nucleopolyhedrovirus (NPV) and granulovirus (GV). The baculovirus life cycle is biphasic. During an early stage of infection the progeny nucleocapsids bud through the host cell plasma membrane to form the budded virus (BV). Later in the infection cycle the progeny nucleocapsids are occluded within proteinaceous matrices called polyhedra in the case of NPVs and granules in the case of GVs. Occlusion derived viruses (ODVs) initiate infection in the insect midgut whereas BVs are involved in the systemic spread of the virus within the insect. Baculoviruses are commonly used as protein expression vectors (Summers, 2006) and as effective and highly selective biopesticides for the protection of field crops and forest (Kamita et al., 2005; Inceoglu et al., 2006).

Significant research has gone into improving the inherent insecticidal activity of the baculovirus by genetic modification (reviewed in Kamita et al., 2005). One highly effective strategy to improve insecticidal activity has been to insert a foreign gene with insecticidal properties (e.g., insect-selective toxin, insect-specific hormone, etc.) at the ecdysteroid UDP-glucosyl transferase (*egt*) gene locus of the NPV genome (O'Reilly and Miller, 1989). The *egt* gene encodes an ecdysteroid glucosyl transferase that catalyzes the conjugation of a sugar molecule to ecdysteroids. This conjugation inactivates the ecdysteroid so that normal eclosion does not occur. The hypothesis is that by preventing normal eclosion the insect host will continue to feed and accumulate additional nutrient resources that will be available to the baculovirus for increased progeny production. In our previous study (Kang et al., 2000), we have shown that deletion of the *egt* gene of BmNPV results in faster virus transmission and improved speed of kill.

In insects, the availability of food plays a critical role in larval growth and subsequent metamorphosis. When food resources are depleted during the juvenile stage, some insects possess evolutionary mechanisms to retard development, whereas others show accelerated metamorphosis (see Cymborowski et al., 1982; Shafiei et al., 2001; Munyiri and Ishikawa, 2005). In *Bombyx mori*, DNA synthesis activity in prothoracic glands is nutrient-dependent during the last larval instar (Chen and Gu, 2006). A shortage of food during this period results in an increase in ecdysteroid production,

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which is hypothesized to help enhance survival. Hoover et al. (1997) have also shown that the nutrient composition of the diet not only affects the relative growth rate of the insect but also affects the median lethal time (LT_{50}) of a baculovirus that infects the insect.

In this study, larvae of the silkworm *B. mori* (5th instar) and tobacco budworm *Heliothis virescens* (1st or 3rd instars) were inoculated (orally with polyhedra or by injection of BV) with wild type or recombinant baculovirus. Following inoculation the larvae were starved for 12 or 24 h before being placed on diet. Alternatively, the inoculated larvae were placed on diet, starved for a 24 h-long period at 1 or 2 days post inoculation (p.i.), and then placed back on fresh diet. The effect of host starvation on baculovirus pathogenicity, larval mortality, and larval mass were quantified.

2. Materials and methods

2.1. Insects and viruses

Eggs of the silkworm *B. mori* and tobacco budworm *H. virescens* were obtained from the National Institute of Agricultural Science and Technology (Republic of Korea) and United States Department of Agriculture, Agricultural Research Service (Stoneville, MS), respectively. Larvae of *B. mori* and *H. virescens* were reared on artificial diet at 26 °C as described previously (Choudary et al., 1995; Hoover et al., 1997). Wild type *Autographa californica* multicapsid nucleopolyhedrovirus (AcMNPV) and AcAaIT, a recombinant AcMNPV carrying the insect-selective scorpion toxin gene AaIT (McCutchen et al., 1991), were propagated on Sf9 cells. Wild type *B. mori* NPV (BmNPV) (Maeda et al., 1985) and BmCysPD, a recombinant BmNPV in which the endogenous cysteine protease gene was replaced with a *lac*Z gene cassette (Ohkawa et al., 1994), were propagated on Bm5 cells. The Sf9 and Bm5 cells were maintained on TC-100 supplemented with 5% and 10% fetal bovine serum, respectively, as described previously (O'Reilly et al., 1992; Choudary et al., 1995). Viral titers were determined by end-point dilution on Bm5 or Sf9 cells as appropriate (O'Reilly et al., 1992).

2.2. Bioassay, starvation regimes, and hemolymph collection

Neonates of *H. virescens* were inoculated with AcMNPV by a droplet feeding method (Hughes et al., 1986). Each droplet (1 μl) contained 2000 PIBs and 5% (v:v) blue food coloring in distilled H_2O. Only larvae that completely ingested the droplet during a 1 h-long exposure were used in the bioassays. Following inoculation, the neonates were starved for 0, 12 or 24 h, and then transferred to fresh diet. Each treatment cohort consisted of 30 larvae. Mortality was checked at 4–6 h intervals until all larvae died. Third instar larvae of *H. virescens*, 30 or 48 larvae per cohort, were inoculated by allowing the larvae to completely ingest a plug of artificial diet (during a 12 h-long exposure period) contaminated with AcMNPV (4000 PIBs) or AcAaIT (4000 PIBs). Following inoculation, the larvae were starved for 0 or 24 h, and then transferred onto fresh diet. Mortality was checked at 4–6 h intervals. The viral doses (2000 PIBs per neonate and 4000 PIBs per 3rd instar) were sufficient to induce 100% mortality by 6 days p.i.

Female larvae of *B. mori*, 1 day after the 4th larval ecdysis, were inoculated by hemocoelic injection of 20 μl of tissue-culture medium containing 2.9×10^4 $TCID_{50}$ of BmNPV or BmCysPD as described previously (Choudary et al., 1995). Following inoculation, the larvae were starved for 0, 12 or 24 h and then placed on fresh diet. Other larvae were placed on diet immediately after inoculation, starved for a 24 h-long period at 1 or 2 days p.i., and then returned to fresh diet. Larval mortality was scored at 4–6 h intervals. Larval mass was determined at 24 h intervals. At 3 or 4 days p.i., the hemolymph was collected into a chilled microfuge tube containing a few crystals of phenylthiourea by piercing a proleg with a sterilized pin as described previously (Choudary et al., 1995). The hemolymph was appropriately diluted and the titer of virus in the hemolymph was determined as described below. Median lethal time to death (LT_{50}) was determined using the POLO prohibit analysis program (LeOra).

2.3. SDS–PAGE and western blot analysis

Hemolymph proteins were separated by SDS-(12%) PAGE as described by Laemmli (1970). The hemolymph sample was pooled from 10 individuals (100 μl from each larva) for each treatment group. Prior to SDS–PAGE, the hemolymph sample was mixed with sample buffer (final concentration of 2% SDS, 6.25 mM Tris–HCl pH 6.7, 15% glycerol and 100 mM dithiothreitol) and boiled for 5 min. Following electrophoretic separation of the proteins, the gel was stained with Coomassie brilliant blue R-250. For western blot analysis, pooled hemolymph proteins from 10 individuals were separated by SDS-(12%) PAGE as described above and then transferred to a nitrocellulose membrane. Following protein transfer, the membrane was blocked with 3% (w:v) gelatin in Tris-buffered saline (TBS) for 1 h, and then incubated for 2 h with mouse anti-polyhedrin antibody (a gift from Prof. Yeon Ho Je, Seoul National University). The mouse anti-polyhedrin antibody was diluted 1:10,000 in TBS containing 0.02% Tween 20 (TBS-T) containing 1% (w:v) gelatin. Following incubation with the first antibody the membrane was washed with TBS-T, and then incubated for 1 h with rabbit anti-mouse alkaline phosphatase-conjugated antibody (Bio-Rad). The rabbit anti-mouse alkaline phosphatase-conjugated antibody was diluted 1:3000 in TBS-T containing 1% (w:v) gelatin. Following incubation with the second antibody the membrane was washed with TBS-T, and then stained using an Alkaline Phosphatase Conjugate Substrate Kit (Bio-Rad) following the manufacturer's protocol.

2.4. Histochemical analysis of the transmission of BmCysPD in 5th instar B. mori

The expression of β-galactosidase, an indicator of BmCysPD transmission in tissues of *B. mori*, was visualized by staining with 5-bromo-4-chloro-3-indolyl-β-D-glactopyranoside (X-gal). At the appropriate time p.i., each larva was secured onto a block of Styrofoam with dissecting pins and a longitudinal incision was made through the dorsal cuticle. The dissected larva or tissue was immersed with PBS (0.5 M sodium phosphate, 0.5 M NaCl, pH 6.0) for 3 min. The PBS was then replaced with fixative solution (4% paraformaldehyde in PBS). After 20 min, the larvae were washed twice with PBS and infused with staining solution [5 mM $K_4Fe(CN)_6$, 5 mM $K_3Fe(CN)_6$, 2 mM $MgCl_2$ in distilled H_2O] containing 2 mg/ml of X-gal and placed in total darkness for 6–12 h as described previously (Engelhard et al., 1994).

2.5. Determination of viral titer

The hemolymph of virus-infected larvae (following starvation for a 24 h-long period immediately following inoculation or at 1 or 2 days p.i.) was collected at 3 or 4 days p.i., into chilled microfuge tubes and centrifuged at 1000×*g* for 5 min to remove hemocytes and other large particles and stored at −80 °C. The viral titers were measured by end-point dilution with serially 10-fold-diluted samples by modifications of the method described previously (O'Reilly et al., 1992).