Pathogenesis of *Autographa californica* multiple nucleopolyhedrovirus in fifth-instar *Anticarsia gemmatalis* larvae

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We have investigated infection and pathogenesis of *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) in *Anticarsia gemmatalis* (velvetbean caterpillar) larvae using a *lacZ* recombinant virus (AcMNPV-hsp70/lacZ) to track the temporal progression of infection in the midgut intestine and haemocoel. *A. gemmatalis* was highly resistant to fatal infection by occlusion bodies (OBs; LD$_{50}$ $= 5.5 \times 10^5$ OB) and budded virus (BV; LD$_{50} > 3\times 10^5$ BV) administered via oral and systemic routes, respectively. Orally administered occlusion-derived virus (ODV) efficiently attached and fused to midgut cells; however, high levels of infection-induced apoptosis limited infection in the midgut. Transcriptional analysis of AcMNPV genes expressed in the midgut of OB-inoculated *A. gemmatalis* larvae showed high levels of mRNA encoding the major capsid protein VP39 in the absence of immediate-early transactivator 1 (*ie-1*) expression. In the midgut, virus was efficiently transferred from infected midgut epithelial cells to nearby tracheolar cells and circulating haemocytes to initiate systemic infection in the haemocoel. However, haemocoelic BV did not efficiently disseminate infection and only cuticular epidermal cells displayed high levels of viral infection. Flow cytometry analysis of haemocytes isolated from BV-inoculated *A. gemmatalis* larvae showed low-level expression of the BV envelope protein GP64 on the cell surface, suggesting that *A. gemmatalis* haemocytes have a limited capacity for amplifying virus. These results show that AcMNPV is not an effective biological control agent for limiting crop damage caused by *A. gemmatalis* larvae.

INTRODUCTION

*Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) mortally infects several species of *Lepidoptera* larvae and is the best-studied nucleopolyhedrovirus in the family *Baculoviridae*. While baculoviruses are effective in controlling crop pests, several factors, including low virulence and narrow effective host ranges, limit their efficacy (Vail, 1993; Vail *et al.*, 1999). However, baculoviruses recycle in nature, resulting in pest control over several seasons (Mulock & Faulkner, 1997), a characteristic many other pest control measures (e.g. chemical pesticides or *Bacillus thuringiensis* toxin) lack. Thus, amplification and persistence in the field are important advantages of baculoviruses over conventional pesticides and many other biological agents that are currently applied to control lepidopteran crop pests.

AcMNPV pathogenesis has been studied in extensive detail for several important lepidopteran crop pests. AcMNPV infection is initiated after permissive larvae consume viral occlusion bodies (OBs), which dissolve in the midgut intestine to release occlusion-derived virus (ODV) (Faulkner *et al.*, 1997; Granados, 1978). The ODV envelope mediates specific attachment to midgut epithelial cells, leading to ODV membrane fusion with the cellular plasma membrane (Faulkner *et al.*, 1997; Haas-Stapleton *et al.*, 2004; Kuzio *et al.*, 1989). Nucleocapsids subsequently enter the cytosol and uncoat in the nucleus to express genes encoding early viral proteins, including the budded virus (BV) envelope protein GP64 (Blissard & Rohrmann, 1989; Goley *et al.*, 2006). Systemic infection is established when progeny BV are released from midgut cells to infect nearby tracheolar cells of the respiratory system or haemocytes of the immune system (Granados & Lawler, 1981; Engelhard *et al.*, 1994). In species permissive to AcMNPV infection,
BV disseminates throughout the insect and virus-encoded enzymes are activated that digest host tissues to facilitate dispersion of progeny OB in the environment (Hawtin et al., 1997; Hom et al., 2002).

Over the course of baculovirus coevolution with their hosts (Herniou et al., 2004), it is clear that insects have developed physiological responses to evade or combat baculovirus infection. The insect Spodoptera frugiperda is susceptible to systemic AcMNPV BV, but limited ODV attachment to midgut cells prevents AcMNPV from efficiently initiating infection of S. frugiperda larvae in nature (Haas-Stapleton et al., 2003, 2005). Once a midgut infection is established, AcMNPV can stimulate an apoptotic cascade so that infected cells are eliminated to limit systemic infection (Washburn et al., 1998; da Silveira et al., 2005; Dougherty et al., 2006). Upon entering the haemocoel, Heliconverpa sea and Manduca sexta can mount a cellular immune response against AcMNPV that limits amplification and dissemination of the virus (Washburn et al., 1996, 2000; Trudeau et al., 2001). To limit the impact of systemic infection, soluble factors are biosynthesized by some insect species to inactivate BV circulating in the haemocoel, recruit haemocytes to infection sites or contribute to killing virus-infected cells (Trudeau et al., 2001; Hirai et al., 2004; Merchant et al., 2008). Insect development also plays an important role in baculovirus pathogenesis, as adult moths display limited susceptibility to baculoviruses. Understanding insect responses that limit baculovirus infection will aid farmers in selecting baculovirus species appropriate for controlling specific insect pests and suggest insect pathways that may be modulated to increase the efficacy of baculoviruses and other biological control agents.

Anticarsia gemmatalis (the velvetbean caterpillar) is an important lepidopteran pest of soybean that is typically controlled with chemical pesticides. Herein, we report that penultimate instar A. gemmatalis larvae were highly resistant to infection by AcMNPV OB and BV. Resistance to oral infection with AcMNPV OB was mediated by reduced viral gene expression in the midgut of A. gemmatalis larvae and high levels of apoptosis in midgut epithelial cells. Systemic resistance to AcMNPV infection resulted from low levels of viral envelope protein (GP64) biosynthesis by haemocytes and infectious BV in the haemocoel.

**METHODS**

**Maintenance and inoculation of larvae.** *A. gemmatalis* and *Trichoplusia ni* eggs were purchased from Benzon Research. Larvae were reared in groups on species-specific diets (Southland Products) as described previously (Haas-Stapleton et al., 2003). Under these rearing conditions, *A. gemmatalis* had six instars while *T. ni* had five. Test larvae were orally or intraheamaecocically (IH) inoculated using a microapplicator (Burkard or KD Scientific) and maintained individually as described previously (Haas-Stapleton et al., 2003).

**Viruses, virus purification, quantification and octadecyl rhodamine B chloride (R18) labelling.** The AcMNPV recombinant AcMNPV-hsp70/lacZ was used exclusively for the experiments described herein. AcMNPV-hsp70/lacZ contains all of the wild-type E2 genes in their natural gene order and the *Escherichia coli* β-galactosidase reporter gene driven by the functionally early and constitutive *Drosophila hsp70* heat shock promoter. AcMNPV-hsp70/lacZ produces mortality in larvae at levels identical to wild-type AcMNPV strain E2 (Engelhard et al., 1994). OBs were isolated from virus-killed *T. ni* cadavers, purified and enumerated as described previously (Summers & Smith, 1987). ODV was isolated, aliquots were labelled with R18 (Invironogen), protein and fluorescence levels were quantified and ODVs were stored as described previously (Haas-Stapleton et al., 2004). Protein concentrations and specific fluorescence values of R18-labelled ODV (ODV$_{18}$) were used to calculate the relative fluorescence units per µg ODV$_{18}$ protein (187.5 for ODV$_{18}$ in this study). BV was harvested from the media of infected SF-9 cells at 3 days post-infection (p.i.). BV titres were determined by immunoplaque assay with SF-9 cells and quantified as p.f.u., as described previously (Volkman & Goldsmith, 1982).

**Bioassays.** Newly moulted or feeding penultimate instar larvae were inoculated orally or IH with increasing concentrations of AcMNPV-hsp70/lacZ OB or BV, respectively, and maintained until pupation or death as described previously (Haas-Stapleton et al., 2003; n=28–32 larvae per treatment). For some experiments, vehicle only (DMSO) or 1 % M2R (Sigma Aldrich) solubilized in vehicle was incorporated into inocula containing identical quantities of OB. To evaluate the effect of including M2R with the AcMNPV inocula on insect mass, the masses of pupae were measured at 10 days p.i.

**Time-course.** Time-course experiments were conducted in newly moulted fifth-instar *A. gemmatalis* larvae (hereafter designated 5º) orally or IH inoculated with 10 600 OB or 10$^5$ p.f.u. BV, respectively. At specific times, 20–25 larvae were killed, the haemolymph was removed to quantify the proportion of LacZ-positive (LacZ$^+$) haemocytes and BV titre, and the tissues were processed for LacZ expression as described previously (Trudeau et al., 2001). Tissues were examined using a stereomicroscope (Leica MZ12.5) to quantify the number and cellular composition of LacZ$^+$ cells. Representative images were captured using a digital camera (MicroPublishing 3.3 RTV, QImaging). At 108 and 132 h p.i. in the OB time-course, 40 % of LacZ$^+$ insects displayed more than 250 LacZ$^+$ cells and were not included in the foci quantity analysis. For the BV time-course, groups of 20 insects per time point were dissected, haemolymph was removed to quantify the proportion of LacZ$^+$ haemocytes and BV titre, and whole mounts of each insect were prepared to quantify the extent of viral infection in the epidermis, malpighian tubule, fat body and tracheal tissues, as described previously (Trudeau et al., 2001). Because IH inoculated BV was disseminated throughout insects, a pathology score (PS) was assigned that correlated with the average range of foci numbers observed for a particular tissue type in a LacZ$^+$ insect using the following scheme: 1=1–10 foci, 2=11–50 foci, 3=50–200 foci, 4=201–500 foci, 5>500 foci. Bioassays were conducted as described above for each time-course to determine mortality levels for inoculated larvae.

**Analysis of haemocyte infection and haemolymph BV titre.** The proportion of LacZ$^+$ haemocytes for each insect in the time-course studies was determined as described previously (Trudeau et al., 2001). The BV titre in haemolymph was quantified with plaque assays based upon the method described by Volkman & Goldsmith (1982), with modifications described by Dee & Shuler (1997). Briefly, haemolymph from each insect was added to a well of a 96-well plate resting on dry ice and subsequently stored at −80 ºC. Haemolymph was thawed by dilution with insect culture media [1: 20; TNMFH media (Lonza BioWhittaker) containing 1-phenyl-2-thiourea (1 % w/v)] and centrifuged for 10 min (260 g); the supernatants were removed and serially diluted in TNMFH media. To determine BV titre, 10 µl
diluted haemolymph was added to each of four wells of a 96-well plate containing 10⁵ SF-9 cells in 90 μl media. Plates were centrifuged for 60 min (1000 g) to synchronize infect cells. Methylicellulose (0.6% w/v) was subsequently added to each well and the plates were incubated at 28 ± 2 °C for 72 h. Cells were processed for LacZ and foci number was enumerated to determine haemolymph BV titre, as previously described (Trudeau et al., 2001).

Apoptosis detection. Fifth-instar A. gemmatalis larvae were orally inoculated with 5 x 10⁵ OB or vehicle (glicerine and water; 3:2 v/v). Larvae were dissected at 24 h p.i., the midguts were removed; lumen contents were removed, rinsed for 5 s twice in ice-cold PBS, and fixed in PBS containing paraformaldehyde (PEA, 2% w/v). Apoptotic cells were detected using a colorimetric terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assay (DeadEnd Colorimetric Tunel System, Promega) according to the manufacturer’s instructions for frozen tissue sections, with the following modifications (n=12–16 larvae per treatment). Midgut tissues were not frozen or sectioned. The tdT reaction mix incubation time was 75 min. Apoptotic cells stained with this TUNEL assay displayed dark brown nuclei that we visualized with a stereomicroscope (Leica MZ12.5) and representative images collected using a digital camera (MicroPublishing 3.3 RTV, QImaging). Some midguts were processed for LacZ signals and imaged as described above (n=10 larvae per treatment). The proportions of LacZ- and TUNEL-positive mature midgut columnar cells in the midgut were estimated using a stereomicroscope to select five fields of view and counting the number of mature midgut columnar cells that were positive or negative for each marker.

ODV<sub>R18</sub> attachment and fusion. ODV<sub>R18</sub> attachment and fusion experiments were performed as described by Haas-Stapleton et al. (2004), with the following modifications. Fifth-instar A. gemmatalis and penultimate (fourth) instar T. ni larvae were inoculated orally with 0.64 μg ODV<sub>R18</sub> (n=29–32 larvae of each species) or vehicle (n=12–16 larvae of each species). Midguts were excised, the lumen contents were removed, tissues were rinsed twice in buffer A (100 mM Na<sub>2</sub>CO<sub>3</sub>, 100 mM KCl, 100 mM EGTA, pH 9.5) and each midgut was frozen in buffer A at −80 °C in a separate well of a 96-well plate. ODV<sub>R18</sub> fluorescence levels were measured as described previously (Haas-Stapleton et al., 2004). The quantity of ODV<sub>R18</sub> attached and fused to cells was calculated using relative fluorescence units per μg ODV<sub>R18</sub> protein. Attached and fused quantities of ODV<sub>R18</sub> were normalized to 1 μg midgut protein.

Gene expression. Fifth-instar A. gemmatalis and 4<sup>th</sup> T. ni larvae were orally inoculated with 5 x 10⁵ OB or vehicle (glicerine and water; 3:2, v/v). Larvae were dissected at 6, 12 and 24 h p.i. in ice-cold PBS, the midguts were removed, opened to remove lumen contents, rinsed twice for 15 s in ice-cold PBS, placed in 2 ml Trizol Reagent (Invitrogen) and frozen at −80 °C (n=12 insects per treatment, experiment repeated three times). Total RNA was isolated and treated with amplification grade DNase I (Sigma Aldrich) according to the manufacturer’s instructions. RNA concentrations and purity were estimated using a spectrophotometer (SmartSpec Plus, Bio-Rad Laboratories). Because viral genes are expressed at different times over the course of an infection, we combined equal quantities of RNA isolated at each time point from virus- or vehicle-inoculated larvae and used this pooled RNA to assess viral gene expression in the midgut with semiquantitative RT-PCR. RNA was reverse-transcribed using the Titan One Tube RT-PCR kit (Roche Applied Science) and primers were designed to amplify AcMNPV ie-1, chla, dnapl, vp39, poly or cellular gapdh with 10 initial PCR cycles followed by 26 PCR cycles, according to the manufacturer’s instructions. Primer sequences, PCR product sizes, and annealing temperatures for reverse transcription and PCR are provided in Supplementary Table S1 (available in JGV Online). Using RNA combined from midguts isolated 6, 12 and 24 h.p.i. from virus-inoculated 5<sup>th</sup> A. gemmatalis, the intensity of the gapdh amplification product increased up to 32 cycles following the initial 10 cycles (Supplementary Fig. S1a, available in JGV Online). PCR products were analysed by agarose gel electrophoresis and imaged using a Gel Doc XR System (Bio-Rad Laboratories). Viral gene expression levels in the midgut of A. gemmatalis and T. ni were normalized to the expression levels of the cellular gapdh gene (presented as the average density (AD) ratio of viral gene to gapdh).

Flow cytometry and microscopy of haemocytes. Fifth-instar A. gemmatalis and newly moulved 4<sup>th</sup> T. ni, were IH inoculated with 10⁵ p.f.u. BV or vehicle (TNMF/H media) and haemolymph was removed from cohorts of 10–15 larvae at 24, 48, 72 and 96 h p.i., as described by Trudeau et al. (2001). Haemolymph was diluted 1:10 in PBS lacking divalent cations (DPBS; Fisher) at 25 °C, fixed for 5 min with DPBS containing 2% PFA (w/v, final), centrifuged (10 min, 260 g 4 °C) and the cells were suspended in DPBS containing 1% (w/v) BSA fraction V (DPBS-BSA; Sigma Aldrich). Haemocytes were washed twice in DPBS-BSA using centrifugation (10 min, 260 g 4 °C), stained with the mouse monoclonal antibody B12D5 specific for GP64 (1:50), rinsed twice in DPBS-BSA and cells were suspended in goat anti-mouse IgG(H + L)-FITC (Southern Biotech; 1:400) to detect binding of B12D5. Stained haemocytes were rinsed twice with DPBS-BSA, suspended in DPBS-BSA containing propidium iodide (PI; 1 μg ml⁻¹; Molecular Probes) to identify intact cells and analysed by flow cytometry using a Quanta SC MPL (Beckman Coulter). Only cells that gated PI-positive were analysed for FITC intensity. Fluorescence and differential interference contrast (DIC) microscopy was used to capture representative images of haemocytes using an Olympus BX51 fitted with an F-View digital camera and Microsuite software (Olympus). Graphical, statistical and image processing software. Data were analysed and graphs were generated using Prism 5.0 (GraphPad Software). Error bars throughout reflect SEM. Densitometry analyses for gene expression studies were made using Quantity One 1-D Analysis Software (Bio-Rad Laboratories). In some cases, images were cropped using Adobe Photoshop CS4 (Adobe Systems) and fluorescence images of haemocytes were coloured using Microsuite software (Olympus).

RESULTS AND DISCUSSION

Relative susceptibility of 4<sup>th</sup> larvae to AcMNPV infection

In this study, we characterized the pathogenesis of the baculovirus AcMNPV in 5<sup>th</sup> A. gemmatalis using AcMNPV-hsp70/lacZ. A. gemmatalis larvae were more than 40,000-fold more resistant to fatal infection by orally inoculated OB compared with T. ni (Fig. 1a). The LD<sub>50</sub> for A. gemmatalis was estimated at 5.5 x 10⁵ OB (the precise LD<sub>50</sub> for OB-inoculated A. gemmatalis was not determined because the highest dosage inoculated generated 47% mortality and more concentrated inocula was too viscous to flow reliably through inoculation needles). Similarly, A. gemmatalis IH inoculated with BV was more than 10⁶-fold more resistant than T. ni (Fig. 1b). The LD<sub>50</sub> for A. gemmatalis IH inoculated with BV was not determined because the maximum dosage inoculated (3 x 10⁵ p.f.u.) caused a final larval mortality of 19%. It is of note that fewer than 4% of OB- or BV-inoculated A. gemmatalis larvae that succumbed to viral infection had liquefied.
Time-course of orally inoculated OB

To determine whether the barrier to OB infection resulted from interactions of the virus with midgut cells or transfer of virus to cells in the haemocoel, we conducted a time-course experiment with *A. gemmatalis* orally inoculated with 10 600 OB. At 14 h p.i., we observed the first signs of AcMNPV infection simultaneously in cells of the midgut epithelium, tracheolar cells associated with infected midgut epithelial cells, and in circulating haemocytes (Fig. 2a). Midgut foci constituted the largest proportion of LacZ+ cells at 14 h p.i. (9.1 ± 2.3 midgut foci per LacZ+ insect; Fig. 2b); however, 43% of the infected larvae also exhibited tracheal infections (6.9 ± 1.8 tracheal foci per LacZ+ insect; Fig. 2b).

Of the LacZ+ larvae at 14 h p.i., 31% harboured foci consisting of only tracheal cells, suggesting that the underlying midgut cells had been shed. At 14 h p.i., infected tracheal cells were associated with underlying infected midgut epithelial cells in 50% of LacZ+ larvae (Fig. 2b) and LacZ+ haemocytes were detected in 14% of LacZ+ larvae (Fig. 2a). These results demonstrate that BV released from infected midgut cells efficiently established systemic infection in nearby tracheolar cells or haemocytes. The observed lack of a delay between the onset of LacZ signalling in the midgut and the haemocoel lends support to the hypothesis proposed by Washburn et al. (2003) whereby nucleocapsids derived from ODV are transported through the cytosol of midgut epithelial cells to the basal plasma membrane and are released as BVs after early GP64 biosynthesis.

Although 10 600 OB were orally inoculated into each larva, a maximum of 21.6 ± 3.7 midgut foci per LacZ+ insect were observed at 24 h p.i. (Fig. 2b; OB: midgut foci ratio = 491), suggesting that midgut epithelial cells were highly resistant to productive infection by OB. At 24 h p.i. and later time points, melanized tracheal branches adjacent to the midgut were detected (see Supplementary Fig. S2a, available in JGV Online). However, the melanized tissues contained neither LacZ+ cells nor haemocytes. LacZ signals were absent from the fat body of all assayed insects, providing evidence that systemic viral infection was limited.

At 36 h p.i., 100% of OB-inoculated larvae were LacZ+, 95% harboured infected tracheal cells and 14% contained LacZ+ haemocytes (Fig. 2a). Additionally, at 36 h p.i., we detected for the first time haemolymph BV in 15% of larvae (average BV titre = 1.0 ± 0.0 × 102 p.f.u. ml−1). At 48 h p.i. the proportion of LacZ+ larvae was smaller, which may be due to the reduced LacZ expression typically observed during the premoult period of AcMNPV-Hsp70/lacZ-infected larvae (Haas-Stapleton et al., 2003; Washburn et al., 1995, 2000). At 132 h p.i., the last time point assayed, 38% of the inoculated larvae had pupated and were not evaluated for LacZ signals because of the high endogenous LacZ signals observed in pupae of vehicle-inoculated larvae (results not shown). For insects in a larval stage at 132 h p.i., 85% harboured a systemic infection (tracheal and/or haemocyte infection). Furthermore, at 132 h p.i., haemolymph BV was detected in 100% of LacZ+ larvae (average BV titre 1.0 ± 0.22 × 104 p.f.u. ml−1). In the accompanying bioassay (n=95 larvae), 23% of the inoculated larvae that failed to eclose as adult moths (62% final mortality) succumbed as pupae (see Supplementary Fig. S2b). In contrast, all vehicle-inoculated larvae successfully eclosed.

### Apoptosis and M2R effect on viral pathogenesis

Ten hours after the first signs of infection (24 h p.i.), we observed a marked increase in the number of viral foci that were composed of only tracheal cells (Fig. 2b), suggesting that an underlying infected midgut cell had been released (Washburn et al., 1998). The release of baculovirus-infected cells from the midgut epithelia is thought to result from infection-mediated induction of apoptosis (Clarke & Clem, 2003; Clem, 2007); this process is inhibited by the chemical M2R (Washburn et al., 1998; Dougherty et al., 2006). To evaluate the importance of infected cell shedding in the midgut of *A. gemmatalis* larvae, we orally inoculated 5th larvae and larvae 16 h post-

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**Fig. 1.** Mortality levels for *A. gemmatalis* (●) or *T. ni* (□) inoculated with OB or BV. (a) Mortality levels for newly moulted 4th larvae inoculated orally with increasing concentrations of OB. The regression equations are: for *T. ni*, $y = 4.850x - 62.59$ ($r^2 = 0.9526$), $LD_{50} = 23$ OB; for *A. gemmatalis*, $y = 0.0000748x + 8.787$ ($r^2 = 0.8834$), $LD_{50} = 5.5 	imes 10^5$ OB. (b) Mortality levels for feeding 4th *A. gemmatalis* or *T. ni* IH inoculated with increasing concentrations of BV. The regression equations are: for *T. ni*, $y = 481.0x + 25.69$ ($r^2 = 0.8869$), $LD_{50} = 0.051$ p.f.u.; for *A. gemmatalis*, $y = 0.0000356x + 8.527$ ($r^2 = 0.7175$), $LD_{50} = 3 	imes 10^5$ p.f.u.
Average number of LacZ+ cyte infection (susceptibility of both 50- and 516-inoculated larvae to fatal addition of 1% M2R to viral inocula increased the

Inoculation of 1% M2R to viral inocula increased the susceptibility of both 50- and 516-inoculated larvae to fatal infection, however, the difference was not statistically significant (Fig. 3a; 64% and 82% mortality, respectively; Fisher’s exact test, P = 0.1829). However, considering the shallow slope of the dose-response curve for OB-inoculated larvae (Fig. 1a), the increased mortality associated with the addition of M2R is substantial.

By 10 days p.i., inoculated insects had succumbed to infection as larvae or had pupated. We observed no significant difference in pupa mass for insects inoculated at the same developmental stage with M2R- or vehicle-containing inocula (Fig. 3b; Student’s t-test of 50-vehicle and 50-M2R, P = 0.8280 and of 516-vehicle and 516-M2R, P = 0.4465). In contrast, we observed a moderate but significant difference in pupa mass between 50- and 516-inoculated larvae [average pupa mass: 50, 0.1350 ± 0.0045 (n = 55); 516, 0.1484 ± 0.0036 (n = 57); 50 versus 516, Student’s t-test: P = 0.0213]. Because 516-inoculated larvae fed for 16 h prior to OB inoculation, these results suggest that infection suppressed insect feeding in the 50-inoculated larvae. A. gemmatalis (50 and 516) inoculated orally with OB containing inocula and vehicle were equally susceptible (Fig. 3a; 49 and 43% mortality, respectively), suggesting that developmental resistance to OB is attenuated in A. gemmatalis larvae.

Because mortality levels for larvae inoculated with M2R-containing inocula were higher for both developmental stages tested (Fig. 3a) and M2R inhibits AcMNPV infection-induced apoptosis in midgut cells (Dougherty et al., 2006), we sought to determine if AcMNPV infection induced apoptosis in the midgut of A. gemmatalis. For this study, we orally inoculated 50 A. gemmatalis with vehicle or 5 × 10^5 OB, dissected larvae at 24 h p.i. and detected apoptotic cells in the midgut using the TUNEL assay (Fig. 3c) or in virus-infected cells by LacZ-staining (Fig. 3d). The TUNEL assay measures nuclear DNA fragmentation, a hallmark of the late stage in an apoptotic cascade, by staining apoptotic nuclei a dark brown colour. The midguts of vehicle-inoculated larvae processed with the TUNEL assay displayed dark-staining nuclei in fewer than 0.1% of mature midgut columnar cells (Fig. 3e). In contrast, more than 15% of the mature midgut columnar cells in AcMNPV-inoculated larvae were apoptotic (dark-brown nuclei in Fig. 3c). Fewer than 1% of mature midgut columnar cells in OB-inoculated larvae displayed LacZ signals (arrowheads, Fig. 3d). Relative to the number of apoptotic cells in the midgut of AcMNPV-inoculated larvae, we observed few LacZ+ cells (Fig. 3d), suggesting that apoptosis was stimulated in midgut cells prior to the accumulation of sufficient quantities of LacZ to visualize infected cells.

**ODV attachment and fusion**

Low oral infectivity of OB has been attributed to inefficient ODV attachment to midgut epithelial cells (Haas-Stapleton et al., 2005). Therefore, we quantified levels of ODV attachment and fusion in A. gemmatalis midgut tissue. T. ni served as a positive control because it is highly susceptible to AcMNPV-hsp70/lacZ OB and ODV (Fig. 1a; Engelhard et al., 1994; Washburn et al., 1995). For ODV_R18-inoculated larvae, there was no significant difference in the amount of ODV_R18 bound or fused per μg midgut protein between A. gemmatalis and T. ni (Fig. 4a; bound: A. gemmatalis, 0.00690 ± 0.00089 and T. ni, 0.0037 ± 0.00098; Student’s t-test, P = 0.3957; fused: A. gemmatalis, 0.0105 ± 0.0011 and T. ni, 0.00875 ± 0.00099, Student’s t-test, P = 0.635). Thus, the observed low rate of AcMNPV infection in the midgut of A. gemmatalis does not result from inefficient interactions of ODV with the plasma membrane of midgut cells.
**Midgut viral gene expression**

To determine whether nucleocapsids deposited into cells after ODV envelope fusion uncoat to produce viral mRNA, we used semiquantitative RT-PCR to measure transcription levels of AcMNPV genes characteristic of immediate (ie-1), early (dnapol), late (chiA and vp39) and very late (polh) stages of viral gene expression (Kool & Vlak, 1993; Hefferon, 2004) in midguts isolated from *A. gemmatalis* and *T. ni* inoculated orally with OB (Fig. 4b). Expression of *ie-1* is reportedly required to trigger apoptosis in cultured insect cells infected with AcMNPV (Schultz et al., 2008); however, we did not detect *ie-1* in midguts isolated from OB-inoculated *A. gemmatalis* larvae (Fig. 4b). *A. gemmatalis* larvae inoculated with the same OB dosage displayed high levels of apoptotic midgut cells (Fig. 3c), suggesting that studies of cultured insect cells do not reflect what occurs during a natural baculovirus infection in insects. We observed low mRNA expression levels of viral DNA polymerase (dnapol) in midguts (Fig. 4b), suggesting that the AcMNPV genome replicates in the midgut and/or

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**Fig. 3.** Analysis of AcMNPV-induced apoptosis in the midgut of *A. gemmatalis* larvae. (a) Mortality levels of 5° and 5°* A. gemmatalis* inoculated orally with 2.5 × 10⁵ OB, with and without the optical brightener M2R. (b) Mass of pupae at 10 days p.i. for 5° or 5°* A. gemmatalis* inoculated orally with 10⁵ OB, with and without the optical brightener M2R. (c–e) TUNEL (c) or LacZ (d) staining in representative midguts of 5° A. *gemmatalis* inoculated orally with 5×10⁵ OB or no virus (e, TUNEL staining only) and dissected at 24 h p.i. Bars, 50 μm.

**Fig. 4.** AcMNPV binding, fusion and gene transcription in the midgut of *A. gemmatalis* and *T. ni* orally inoculated with AcMNPV- hsp70/lacZ. (a) Quantity of ODV₉₁₈ bound (black bars) or fused (grey bars) per μg midgut protein of newly moulted 4° larvae inoculated orally with 0.64 μg ODV₉₁₈. (b) Semiquantitative RT-PCR analysis of insect and viral gene expression in midguts isolated from *A. gemmatalis* (Ag) or *T. ni* (Tn) larvae orally inoculated with 5×10⁵ OB. Relative mRNA abundance was quantified by densitometry and expressed as the average density (AD) ratio of viral gene : gapdh for both species.
associated tissues of *A. gemmatalis*. Additionally, moderate levels of the mRNA encoding polyhedrin (*polh*) were detected, although OBs were not observed in any midgut epithelial cells examined in this study. In the RNA isolated from midguts of negative-control vehicle-inoculated *A. gemmatalis* and *T. ni,* only *gapdh* expression was detected (results not shown).

High levels of *vp39* mRNA accumulated over time in the midgut of OB-inoculated *A. gemmatalis* (Fig. 4b and Supplementary Fig. S1b). VP39 is the major capsid protein of AcMNPV (Summers *et al.*, 1978) and the presence of this mRNA is intriguing because its expression in the midgut appears to be independent of IE-1 or other factors transactivated by IE-1. IE-1 is known to be a viral transcription factor that initiates the cascade of AcMNPV gene expression leading to VP39 biosynthesis. The absence of *ie-1* expression in the midgut of AcMNPV-infected *A. gemmatalis* (Fig. 4b), late *vp39* expression may have triggered the observed apoptosis in the midgut (Figs 3 and 4b), as has been shown for capsid protein in other viruses including reovirus, West Nile virus, coxsackievirus B3, parvovirus B19 and bluetongue virus (Henke *et al.*, 2000; Mortola *et al.*, 2004; Poole *et al.*, 2004; Clarke *et al.*, 2005; Yang *et al.*, 2008).

**Time-course of IH inoculated BV**

To understand the molecular process that limits AcMNPV infection in the haemocoel of *A. gemmatalis* (Fig. 1b), we studied the course of infection for 5° *A. gemmatalis* IH inoculated with 10⁵ p.f.u. BV (Fig. 5). Representative images of whole mounts for LacZ⁺ larvae display the extent of LacZ expression observed throughout the IH time-course study (Supplementary Fig. S2c). The extent of viral infection is reported as the average pathology score (APS), scaled from 1 to 5. For tracheal tissues, the APS remained low throughout the time-course, rising to only 0.45 ± 0.28 at 96 h p.i. (Fig. 5b); in malpighian tubules and fat body tissues, the APS was moderately higher (0.16–1.32 ± 0.09–0.38). In contrast, epidermal tissues displayed relatively high APS at all time points after 5 min p.i., with peaks observed at 12 and 96 h p.i. (3.80 ± 0.24 and 2.58 ± 0.24, respectively). This suggests that epidermal cells in *A. gemmatalis* larvae are relatively susceptible to AcMNPV infection and may serve as a reservoir for the virus during the course of infection.

At 5 min p.i., we did not observe LacZ⁺ cells in any larvae (Fig. 5c); however, BV was detected in the haemolymph of each larva sampled (Fig. 5c; 8.92 ± 1.08 × 10⁵ p.f.u. ml⁻¹). While over 80% of BV-inoculated larvae displayed LacZ⁺ cells from 4 to 72 h p.i. (Fig. 5a), haemolymph BV was not detected again until 72 h p.i. (Fig. 5c; 0.45 ± 0.044 × 10⁵ p.f.u. ml⁻¹). At 96 h p.i., high quantities of haemolymph BV were detected in 88% of LacZ⁺ larvae (Fig. 5c; 5.80 ± 2.86 × 10⁵ p.f.u. ml⁻¹). The percentage of LacZ⁺ haemocytes in insects remained relatively low (<1.15%)

![Figure 5](http://vir.sgmjournals.org)
except at 12 and 96 h p.i., when the proportions of LacZ+ haemocytes were elevated (1.68 ± 0.22 % and 5.59 ± 3.92 %, respectively). Low levels of haemolymph BV and LacZ+ haemocytes indicate attenuated BV production in virus-infected cells for several days following inoculation or soluble factors in the haemolymph that may inactivate BV. While these hypotheses are not mutually exclusive, the latter has been observed in bacteria infecting M. sexta (Eleftherianos et al., 2007) and thus may be of interest for future studies of AcMNPV-infected A. gemmatalis larvae.

**GP64 distribution on haemocytes**

We observed a coincident increase in the proportion of LacZ+ haemocytes and BV titre in A. gemmatalis larvae IH inoculated with BV (Fig. 5c). To determine whether haemocytes can produce viral proteins requisite for progeny BV, we used flow cytometry and microscopy to study the expression of GP64 on the surface of haemocytes isolated from A. gemmatalis and T. ni IH inoculated with 10^5 p.f.u. BV or vehicle (Fig. 6). GP64 is a viral protein localized to the BV envelope that is essential for BV budding from infected cells (Oomens & Blissard, 1999), attachment to cells for establishing infection (Hefferon et al., 1999) and cell-to-cell dissemination of virus (Monsm et al., 1996). Relative to haemocytes isolated from T. ni IH inoculated with BV, we observed low levels of GP64 on haemocytes isolated from BV-inoculated A. gemmatalis larvae (Fig. 6). Low level expression of GP64 on the surface of A. gemmatalis haemocytes would substantially reduce the quantity of BV produced by these mobile cells and may be responsible for the limited amplification and dispersal of AcMNPV infection observed in the haemocoel of AcMNPV-inoculated A. gemmatalis larvae (Figs 2a, b and 5b; Supplementary Fig. S2a, c).

AcMNPV is the type species in the family Baculoviridae and is the most extensively studied baculovirus. Consequently, much of the work aiming to genetically improve baculoviruses to enhance their utility for crop protection has been...
performed using AcMNPV. A. gemmatalis is a significant lepidopteran pest of soybean crops, but our results show it to be resistant to fatal infection by AcMNPV OB and BV. Thus, many of the genetic improvements made to AcMNPV may be of little use for controlling A. gemmatalis outbreaks in the field.

ACKNOWLEDGEMENTS

This work was supported by the College of Natural Sciences and Mathematics at California State University, Long Beach and a grant from the USDA (CREES 2008-03390). We thank Dr Mason Zhang, California State University, Long Beach, USA, for the use of his Olympus BX51 epifluorescence microscope and Dr Jan Washburn, University of California, Berkeley (now Emeritus), for pre-submission review of the manuscript. AcMNPV-hsp70/lacZ and the antibody B12D5 were kind gifts of Dr Loy Volkman, University of California, Berkeley (now Emeritus).

REFERENCES


