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**Early pathogenesis of *Autographa californica multiple nucleopolyhedrovirus* and *Helicoverpa zea single nucleopolyhedrovirus* in *Heliothis virescens*: a comparison of the 'M' and 'S' strategies for establishing fatal infection**

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**Nucleopolyhedroviruses (NPVs) (*Baculoviridae*) produce fatal infections in larval lepidopteran insects. NPVs are designated SNPVs or MNPVs based on whether the occlusion-derived virus (ODV) that initiates primary midgut infections contains single (S) or multiple (M) nucleocapsids. The principal consequence of this ODV packaging is that primary target cells infected with the M phenotype receive multiple nucleocapsids, whereas those infected by the S phenotype receive only one. To determine the biological significance of this difference in the initial infection strategy, a comparison of the primary and secondary infection patterns of the recombinants *Helicoverpa zea* SNPV (*HzSNPV-hsp70/lacZ*) and *Autographa californica* MNPV (*AcMNPV-hsp70/lacZ*) in orally inoculated larvae of *Heliothis virescens* was carried out. At dosages yielding similar final mortalities (~85 %), primary midgut infections by *HzSNPV-hsp70/lacZ* (indicated by *lacZ* expression) were observed 6 h earlier and in greater numbers than those generated by *AcMNPV-hsp70/lacZ*. Infection of secondary target cells in the tracheal epidermis, however, occurred at the same time and at the same rate for both NPVs. A 2 h delay was observed between the onset of primary and secondary *AcMNPV-hsp70/lacZ* infection, supporting the hypothesis that early tracheal infections were initiated by ODV nucleocapsids repackaged as budded virus. In contrast, an 8 h delay was observed with *HzSNPV-hsp70/lacZ*, suggesting that systemic infections were established only after virus replication in primary targets. Significant numbers of both MNPV- and SNPV-infected primary target cells were sloughed from the midgut beginning as early as 16 h post-infection. Midgut cell sloughing may be an important host-mediated selection pressure influencing the evolution of NPV morphology and gene regulation, shaping, in part, baculovirus infection strategies.**

## INTRODUCTION

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Nucleopolyhedroviruses (NPVs) in the family *Baculoviridae* produce fatal infections in larval lepidopteran insects. NPVs are unusual in that two viral forms are necessary to complete the infection cycle *in vivo*. An occluded form (occlusion-derived virus or ODV) infects midgut cells after ingestion by a susceptible host, and a budded form (budded virus or BV) subsequently transmits infection from cell to cell. Both forms are enveloped and genetically identical but are structurally different and enter host cells by different mechanisms. Occlusions, composed primarily of crystalline protein (polyhedrin), help protect the virions from environmental degradation but readily dissolve in the alkaline digestive juices of the host, liberating the ODV. Primary infections, initiated within midgut epithelial cells, produce BV that bud from the basal plasma membrane and spread infection among tissues of the host, which, in turn, produce both BV and occlusions. Spread of BV throughout the tissues of the insect typically takes 4 or more days, leading to death and liquefaction, the hallmark of polyhedrosis disease. Liquefaction liberates millions of occlusions that transmit infection to new hosts.

Historically, species within the genus *Nucleopolyhedrovirus* have been designated as either ‘M’ or ‘S’, referring to whether the ODV particles contain multiple (M) or single (S) nucleocapsids. In contrast, the BV of both SNPVs and MNPVs are packaged singly. The biological consequence of the M phenotype is that the midgut cells of the host are infected with multiple nucleocapsids contained within the ODV envelope. There are many more species of MNPV than SNPV described (Vail *et al.*, 1999; Volkman, 1997), and all known MNPVs infect species in the Lepidoptera, the most recently derived insect order. In addition to lepidopterans, however, SNPVs have been isolated also from species in the orders Hymenoptera and Diptera. These host affinities suggest that the progenitor *Nucleopolyhedrovirus* was probably an SNPV (Rohrmann, 1986) and indicate that the M phenotype is a more recently acquired baculovirus trait. While very little is known about what determines the number of nucleocapsids packaged per virion for the MNPVs, the evolutionary history and host ranges of the NPVs suggest that the M phenotype evolved from the S phenotype and, therefore, may incur a selective advantage.

To determine if there is a functional significance between these two ODV phenotypes during the early stages of infection, we compared virus pathogenesis and infection kinetics of an MNPV, *Autographa californica* MNPV (AcMNPV), and an SNPV, *Helicoverpa zea* SNPV (HzSNPV), in larvae of *Heliothis virescens* (Lepidoptera: Noctuidae). AcMNPV is the type species of the *Nucleopolyhedrovirus* genus (Blissard *et al.*, 2000) and can infect at least 32 lepidopteran species within 12 families (Granados & Williams, 1986); this broad host range shows that AcMNPV possesses an effective strategy for fatally infecting larval lepidopterans. In contrast, HzSNPV is reported to have a relatively narrow host range, encompassing species in the Heliothinae, a subfamily within the Noctuidae which includes *H. virescens* (Allen & Ignoffo, 1969; Getting & McCarthy, 1982; Granados & Williams, 1986; King & Coleman, 001-8701 © 2003 SGM

1989; Mitter *et al.*, 1993; Black *et al.*, 1997). HzSNPV also was the first baculovirus pesticide to be introduced into the commercial marketplace for control of lepidopteran pests (Black *et al.*, 1997), and although commercialization failed, basic research has shown that HzSNPV pathogenesis is similar to that of AcMNPV in noctuid larvae (Granados, 1978; Granados & Williams, 1986; Washburn *et al.*, 2001).

To compare virus pathogenesis *in vivo*, we used recombinants of AcMNPV and HzSNPV containing identical *hsp70/lacZ* reporter-gene cassettes and a host insect fully permissive for both NPVs, newly moulted fourth instar larvae of *H. virescens*. With dosages that yielded similar levels of larval mortality, we conducted time-course experiments comparing the strategies of the two pathogens for establishing primary and secondary infections *in vivo*. We also examined their BV infection kinetics in a primary culture of *H. virescens* haemocytes. Our findings revealed that *H. virescens* haemocytes infected *in vitro* by AcMNPV-*hsp70/lacZ* or HzSNPV-*hsp70/lacZ* have similar infection kinetics. In orally infected *H. virescens* larvae, the kinetics of primary and secondary infection were also similar, although the onset by AcMNPV-*hsp70/lacZ* in midgut cells lagged behind HzSNPV-*hsp70/lacZ* by 6 h. Notably, secondary infection of tracheolar cells was initiated at the same time, 12 h post-inoculation (p.i.), suggesting a midgut transit time of 2 h for AcMNPV and 8 h for HzSNPV. The infection strategies of both NPVs resulted in the timely movement of BV into the tracheal epidermis, countering the host's response of avoiding mortal infection by sloughing infected midgut cells.

## METHODS

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**Insects.** *H. virescens* larvae used in these experiments were reared from eggs provided by DuPont Agricultural Products or purchased from Benzon Research. Larvae were fed a wheatgerm-based diet (modified Stoneville) (Washburn *et al.*, 1995) and maintained at 28±3 °C under constant light through the third instar. Non-feeding, quiescent third instar larvae that were preparing to moult were culled and stored at 7 °C for as long as 18 h in order to synchronize developmental rates and to make available large numbers of test insects of the same age for our experiments (Washburn *et al.*, 1995).

**Virus preparation and quantification.** AcMNPV-*hsp70/lacZ* (E2 parental strain) (Engelhard *et al.*, 1994) and HzSNPV-*hsp70/lacZ* (Elcar HzSNPV-1 parental strain) (Washburn *et al.*, 2001) were used for the experiments described herein. These constructs contain all of the wild-type viral genes from their respective parental strains and the *Escherichia coli* β-galactosidase gene driven by the *Drosophila hsp70* promoter. In cultured insect cells infected with either AcMNPV-*hsp70/lacZ* or HzSNPV-*hsp70/lacZ*, *lacZ* is expressed along with early viral genes, permitting us to monitor infection in host tissues by assaying for the presence of the enzyme. Occlusions of AcMNPV-*hsp70/lacZ* and HzSNPV-*hsp70/lacZ* were isolated from liquefied cadavers of *H. virescens* and partially purified by centrifugation on sucrose gradients

(Summers & Smith, 1987). Subsequently, occlusions were pelleted and resuspended in water, then diluted in a neutrally buoyant glycerin/water solution (3:2, v/v) (Washburn *et al.*, 1995). In bioassays with *H. virescens* larvae, both recombinants produced mortalities equivalent to their parental wide-type strains when administered orally with the same number of occlusions (data not shown). The occlusion dilutions used in this study were stored at 4 °C in the dark and quantified using a haemocytometer (minimum of five counts for each inoculum).

**Comparative replication kinetics.** Because there are no available cell lines that can be infected by both AcMNPV and HzSNPV, we used a primary cell culture of haemocytes from larval *H. virescens* to compare virus replication kinetics *in vitro*. Haemocytes were isolated according to Pech *et al.* (1994) with some modifications. All centrifugation steps were performed at 4 °C. Haemocytes were pelleted by centrifugation in an Eppendorf 5417 C centrifuge at 334 *g* for 8 min. After incubation in anticoagulant buffer (0.098 M NaOH, 0.186 M NaCl, 0.017 M EDTA and 0.041 M citric acid, pH 4.5), haemocytes were pelleted (260 *g* for 3 min) and washed twice in Excell-400 medium (JRH Sciences) by centrifugation. The final pellet was resuspended in 50 µl Excell-400 medium, and cell concentrations were estimated with a haemocytometer. To quantify BV production by haemocytes,  $5 \times 10^3$  cells were placed in triplicate wells of four replica plates already containing 70 µl TC100 medium plus 20 % FBS and AcMNPV-*hsp70/lacZ* or HzSNPV-*hsp70/lacZ* at an m.o.i. of 10 p.f.u. per cell. Cells were infected for 2 h at room temperature, and viral inocula removed and replaced with ice-cold medium. Cells were rinsed twice more by centrifugation (200 *g* for 3 min) in a Beckman GPR tabletop centrifuge. After the final centrifugation step, we added 80 µl of medium to each well; we then removed 10 µl of medium from each well to determine the amount of residual inoculum. Haemocyte cultures were maintained at 28 °C, and at 12, 24, 48 and 72 h p.i., 40 µl of medium were removed from three wells of a replica plate and transferred to individual 500 µl microfuge tubes. Tubes were held at 4 °C until all samples were collected, and plaque assays were performed with the appropriate cell line: Sf9 cells for AcMNPV-*hsp70/lacZ* (Engelhard *et al.*, 1994) and AM1 cells for HzSNPV-*hsp70/lacZ* (McIntosh *et al.*, 1981). Significantly, we determined that *H. virescens* haemocytes gave the same results in plaque assays of AcMNPV-*hsp70/lacZ* as did Sf9 cells, and also HzSNPV-*hsp70/lacZ* as did AM1 cells (D. Trudeau, unpublished data).

Mini time-course experiments were conducted to determine the onset of *lacZ* expression *in vitro*. Primary haemocyte cultures were infected at an m.o.i. of 10 and sampled at 1 h intervals for 5 h; samples were processed for *lacZ* expression as described previously (Engelhard *et al.*, 1994) and examined for blue signals of expression.

**Determination of ODV per occlusion.** The numbers of ODV per occlusion of AcMNPV-

*hsp70/lacZ* and HzSNPV-*hsp70/lacZ* were quantified by transmission electron microscopy (TEM). Occlusions of both recombinants were prepared for TEM following standard protocols. To assess the number of ODV per occlusion, for each virus we printed photographs of sections (15000×) on standardized paper and cut out the images from ~100 different occlusions. The paper image of each occlusion cross section was then weighed, and the number of visible ODV was recorded. Weight provided an accurate measure of occlusion cross-sectional area (linear least-squares regression: AcMNPV-*hsp70/lacZ*,  $r^2=0.94$ ; HzSNPV-*hsp70/lacZ*,  $r^2=0.99$ ) and, therefore, allowed a correlation between occlusion cross-sectional area (wt) and number of ODV.

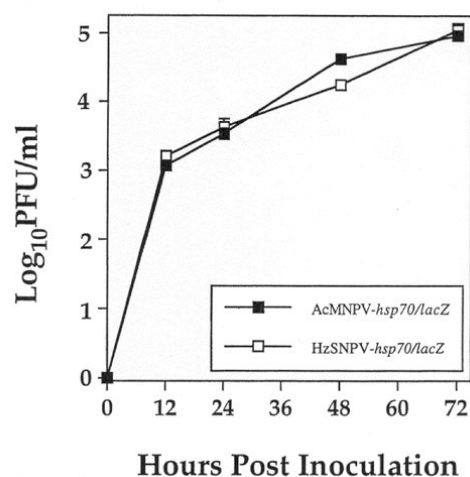
**Bioassay and time-course experiments.** For oral bioassays and time-course experiments, occlusion suspensions in 1.0 µl aliquots were inoculated through the mouth into the lumen of the anterior midgut of newly moulted (i.e. within 15 min of shedding the third instar cuticle) fourth instar *H. virescens* using a syringe fitted with a 32 gauge blunt-tip needle mounted on a microapplicator (Burkard Scientific). After inoculation, larvae were maintained in a growth chamber at 28±2 °C under constant illumination in individual 25 ml plastic cups containing diet *ad libitum*. To determine dosages for time-course experiments, we conducted bioassays in which cohorts of 32 or more larvae were inoculated as described above using varying occlusion numbers of AcMNPV-*hsp70/lacZ* or HzSNPV-*hsp70/lacZ* and maintained until death or pupation. Dosages of 15 and 20 occlusions of HzSNPV-*hsp70/lacZ* and AcMNPV-*hsp70/lacZ*, respectively, were selected for the experiments described here; in four replicated bioassays, 15 occlusions of HzSNPV-*hsp70/lacZ* produced an average mortality of 84 % (range=75–94 %), and 20 occlusions of AcMNPV-*hsp70/lacZ* yielded an average larval mortality of 87 % (range=86–88 %). To assess larval susceptibility to systemic infection, we injected BV of the recombinants directly into the haemocoel of fourth instar larvae using a 32 gauge sharp-tipped needle, as described previously (Engelhard *et al.*, 1994; Engelhard & Volkman, 1995; Washburn *et al.*, 1995).

For time-course experiments, we inoculated large numbers of newly moulted fourth instar *H. virescens* larvae with AcMNPV-*hsp70/lacZ* or HzSNPV-*hsp70/lacZ*. At various times during the first 24 h p.i., cohorts consisting of 26–33 larvae were sacrificed, and their midgut and associated tissues were removed and assessed for *lacZ* expression (Engelhard *et al.*, 1994; Washburn *et al.*, 1995). These tissue preparations were examined for *lacZ* expression with stereo (10–50×) and/or compound microscopy (100–480×) to quantify foci of infection and to identify infected cell types (Washburn *et al.*, 1995, 2001). For each time-course experiment, an additional cohort of 32 insects was retained as an internal control bioassay to confirm mortality levels. We conducted several time-course experiments with each of the recombinants, and the results were the same among experiments. The data used for the analyses of virus pathogenesis presented here were from experiments in which the tissues of 278 and 266 *H. virescens* challenged with AcMNPV-*hsp70/lacZ* and HzSNPV-*hsp70/lacZ*, respectively, were examined for *lacZ*

expression.

## RESULTS

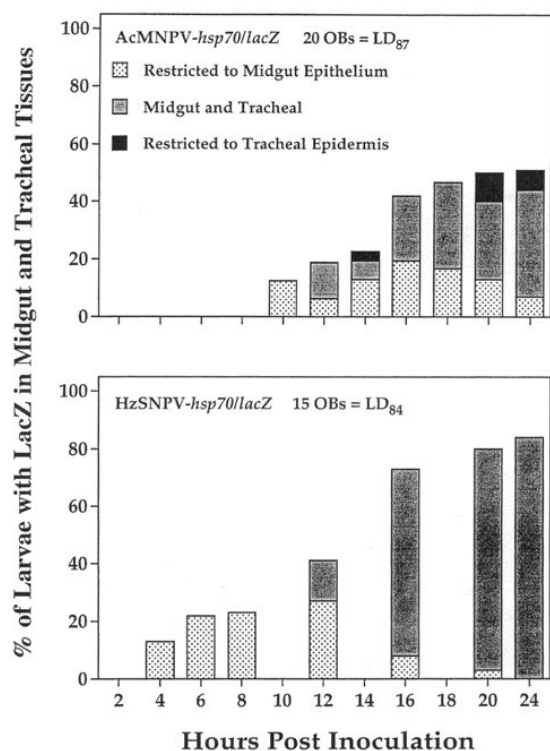
Because AcMNPV-*hsp70/lacZ* and HzSNPV-*hsp70/lacZ* are considerably genetically different, we compared the timing of *lacZ* expression and BV production *in vitro* in haemocytes isolated from *H. virescens*. No LacZ signals were observed in mock-infected haemocytes. We found evidence of *lacZ* expression by 2 h p.i. in cells infected by either virus, but the percentage of cells expressing *lacZ* was lower for the AcMNPV recombinant (0.66 versus 74 % for HzSNPV-*hsp70/lacZ*). By 4 h p.i., *lacZ* expression had increased to 14 % in AcMNPV-*hsp70/lacZ*-infected cells, while in HzSNPV-*hsp70/lacZ*-infected cells, the percentage remained the same. These results suggested that onset of early viral gene expression (as indicated by *lacZ* expression) occurred measurably earlier for the HzSNPV recombinant. Comparison of BV progeny production in one-step growth curves, however, showed that the replication kinetics were nearly identical (Fig. 1), suggesting that, at least by 12 h p.i., the slight lag in AcMNPV-*hsp70/lacZ* gene expression did not measurably retard BV production relative to that of the HzSNPV recombinant.



**Fig. 1.** *In vitro* infection of *H. virescens* haemocytes by AcMNPV-*hsp70/lacZ* and HzSNPV-*hsp70/lacZ*. Haemocytes were infected with an m.o.i. of 10 p.f.u. per cell (determined on *H. virescens* haemocytes), and samples were collected at 0, 12, 24, 48 and 72 h p.i. for BV titre determination. BV titres for AcMNPV-*hsp70/lacZ* and HzSNPV-*hsp70/lacZ* were determined on Sf9 and AM1 cells, respectively. Each data-point represents the mean of three experiments, and error bars represent 1 SE.

Having demonstrated that the two genetically diverse recombinants had similar replication kinetics *in vitro*, we next compared the progression of infection temporally and spatially (as indicated by *lacZ* expression) in cohorts of *H. virescens* larvae inoculated orally with AcMNPV-*hsp70/lacZ* or HzSNPV-*hsp70/lacZ* occlusions (Fig. 2). In these time-course experiments, primary infection of midgut columnar epithelial cells was first detected at 10 and 4 h p.i., respectively, for larvae challenged with the recombinant AcMNPV and HzSNPV viruses. We observed no *lacZ* expression at 9 and 3 h p.i. in larvae inoculated with the MNPV and SNPV, respectively, showing that HzSNPV-*hsp70/lacZ* viral gene expression in the nuclei of the primary cellular targets was initiated 6 h prior to those infected by AcMNPV-*hsp70/lacZ*. At the dosages administered in these studies, *lacZ* expression in secondary target cells within the tracheal epidermis servicing the midgut was observed beginning at 12 h p.i. for both

viruses (Fig. 2). In additional experiments using a dose of 1000 HzSNPV-*hsp70/lacZ* occlusions (approximately 67 times LD<sub>84</sub>), we observed tracheal *lacZ* expression in some larvae as early as 10 h p.i., demonstrating a minimum lag time of 6 h between the onset of primary and secondary infection. When we increased the dosage of the MNPV to 1000 occlusions (approximately 50 times LD<sub>87</sub>), the onset of signalling in midgut and tracheal tissues was the same as with the lower dosage. Thus, the temporal lag between the initiation of primary and secondary infection of *H. virescens* by AcMNPV-*hsp70/lacZ* at both doses was only 2 h (Fig. 2). Bioassays in which we inoculated BV directly into the larval haemocoel revealed that *H. virescens* was exquisitely sensitive to systemic infection by both HzSNPV-*hsp70/lacZ* and AcMNPV-*hsp70/lacZ*. With either virus, BV dosages of 0.01 and 0.1 p.f.u. yielded final larval mortality levels of 20–35 % and 70–82 %, respectively. Generation of such high mortality following injection of extremely low dosages of BV [e.g. for AcMNPV, 1 p.f.u.=2.8 particles (Volkman *et al.*, 1976)] demonstrates that *H. virescens* lacks systemic resistance to infection by these baculoviruses.

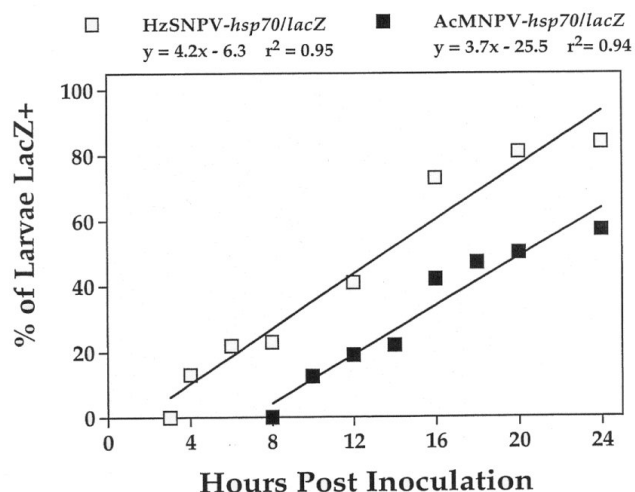


**Fig. 2.** Early pathogenesis of AcMNPV-*hsp70/lacZ* (upper panel) and HzSNPV-*hsp70/lacZ* (lower panel) in *H. virescens* larvae following inoculation of newly moulted fourth instars. Each bar represents a cohort of between 26 and 33 larvae. Occlusion dosages and mortalities represent means determined from five haemocytometer counts and four bioassays, respectively.

Following the onset of tracheal infections, the proportions of systemically infected larvae increased progressively in cohorts inoculated with either HzSNPV-*hsp70/lacZ* or AcMNPV-*hsp70/lacZ*. The greatest proportion of HzSNPV-*hsp70/lacZ*-inoculated larvae with infection restricted to the midgut (29 %) occurred at 12 h p.i., but by 16 h p.i. the proportion had decreased to 8 %. At 24 h p.i. the proportion of LacZ-positive larvae in the cohort (84 %) was equivalent to the final average mortality determined from the bioassays, and all LacZ-positive larvae had one or more foci containing infected tracheal cells. These findings indicated that beyond 24 h p.i., no additional functional primary foci of infection (i.e. that

would lead to death of additional hosts) would be established. In contrast, for AcMNPV-*hsp70/lacZ*-infected insects, the greatest proportion of larvae with infection restricted to midgut cells occurred at 16 h p.i. (19 %), and this proportion decreased gradually thereafter until 24 h p.i. when 8 % of the infected larvae had only midgut-restricted foci. Also, by 24 h p.i. only 57 % of the larval cohort inoculated with AcMNPV-*hsp70/lacZ* was positive for LacZ, indicating that early viral gene expression had not yet occurred in 20 % of the larvae that would eventually succumb. Together, these results suggested that functional primary infections occurred in AcMNPV-*hsp70/lacZ*-inoculated larvae much later into the instar (beyond 24 h post-moult) than in HzSNPV-*hsp70/lacZ*-inoculated larvae (Washburn *et al.*, 1995). At 14 h p.i. and later, we found some larvae inoculated with AcMNPV-*hsp70/lacZ* in which the reporter signal was restricted to tracheal epidermal cells and haemocytes, indicating that all of the infected primary target cells in these insects had been sloughed from the midgut epithelium (Washburn *et al.*, 1998). No examples of this phenomenon were observed within any of the HzSNPV-*hsp70/lacZ*-inoculated cohorts.

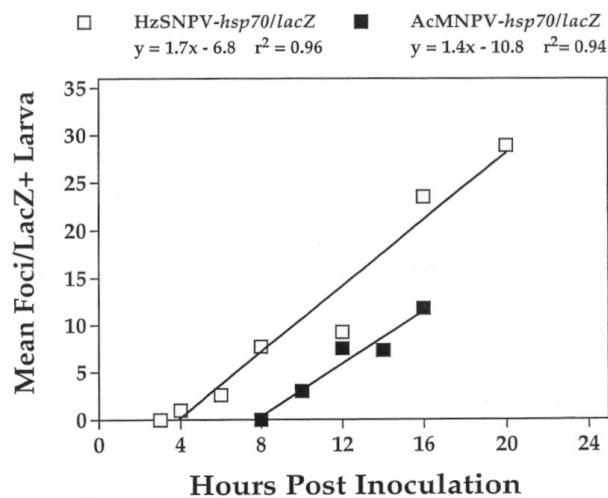
In order to compare the kinetics of larval infection by AcMNPV- *hsp70/lacZ* and HzSNPV-*hsp70/lacZ*, we used least-squares regression to characterize the temporal patterns of *lacZ* expression in cohorts of *H. virescens*. We found that for each recombinant, the temporal increase in the proportion of LacZ-positive larvae during the first 24 h p.i. was well described by a simple linear equation (Fig. 3). While there was a 6 h difference in the onset of midgut *lacZ* expression in larvae inoculated with AcMNPV-*hsp70/lacZ* relative to those inoculated with HzSNPV-*hsp70/lacZ*, the similarity in slope values (ratio of 1.1:1) indicates that (after adjusting for the 6 h difference in initial onset) initiation of infection of *H. virescens* larvae by both viruses proceeded at comparable rates.



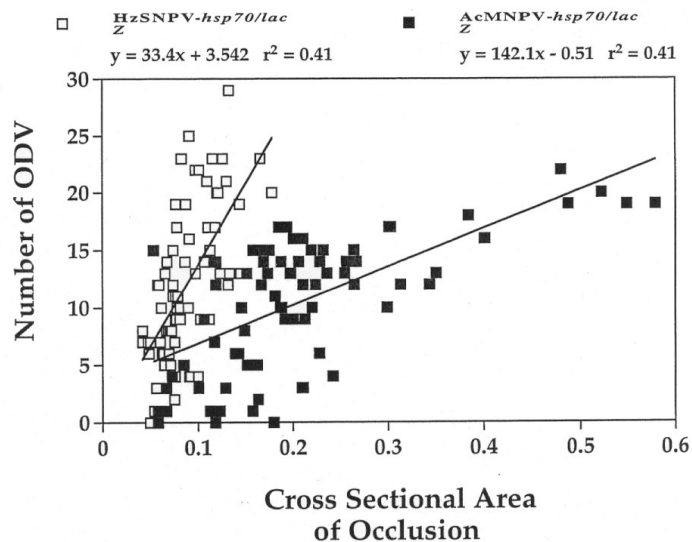
**Fig. 3.** Percentages of *H. virescens* larvae positive for *lacZ* at various h p.i. with either 20 occlusions of AcMNPV-*hsp70/lacZ* or 15 occlusions of HzSNPV-*hsp70/lacZ*. Regression lines were determined by the method of least squares. Each point represents the proportion of LacZ-positive larvae from a cohort of between 26 and 33 larvae.

Early during the infection of hosts, numbers of viral foci are modulated by three dynamic processes: (1) establishment of primary foci of infection in the midgut by ODV; (2) establishment of infected tracheal cells in association with primary foci by BV produced within the primary foci; and (3) loss of primary target cells due to sloughing of midgut cells infected by ODV. While we were unable to measure these

three processes independently, the temporal change in foci numbers and their cellular composition revealed much about the kinetics of primary and secondary AcMNPV and HzSNPV infections. In the time-course experiments illustrated in Figs 2 and 3, we observed maximum numbers of AcMNPV and HzSNPV foci of  $11.8 \pm 4.4$  (mean  $\pm$  1 SE) and  $28.9 \pm 6.4$ , respectively, at 16 and 20 h p.i. (Fig. 4); after these time-points, numbers of foci for both pathogens declined (data not shown). These data show that at doses that achieved the same level of mortality in *H. virescens* larvae, HzSNPV-*hsp70/lacZ* generated significantly greater numbers of viral foci than AcMNPV-*hsp70/lacZ*. They also indicate that, on average, 1.93 viral foci were produced by each occlusion of HzSNPV-*hsp70/lacZ* (e.g. 28.9 foci per 15 occlusions), compared to only 0.59 for AcMNPV-*hsp70/lacZ* (e.g. 11.8 foci per 20 occlusions). Our electron microscopy analyses of the two recombinants revealed a linear relationship between occlusion size and the number of ODV within occlusions (Fig. 5). Overall, the occlusions of the SNPV were smaller than those of the MNPV, but they contained greater numbers of ODV, explaining, at least in part, its higher ratio of foci per occlusion. For both viruses, the temporal increase in foci was linear from the onset of detectable primary infection to the time-point when maximum numbers were observed (i.e. 16 and 20 h p.i. for AcMNPV-*hsp70/lacZ* and HzSNPV-*hsp70/lacZ*, respectively) (Fig. 4). Regression equations for these lines show that the infection of primary cellular targets by AcMNPV-*hsp70/lacZ* was delayed by 6 h relative to HzSNPV-*hsp70/lacZ*, but when the onset times were normalized, midgut cells were infected by both NPVs at similar rates (ratio of slopes=1.2:1).

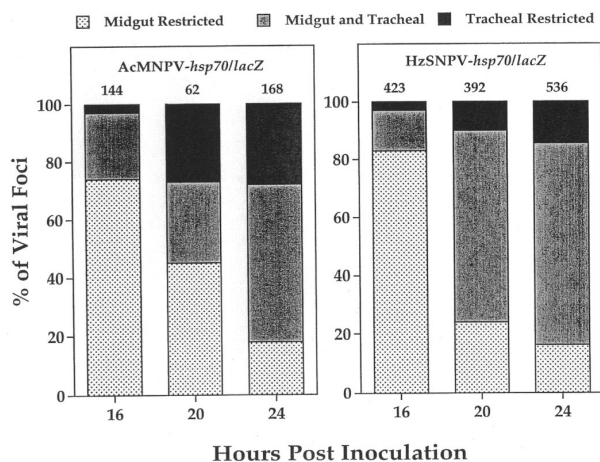


**Fig. 4.** Mean numbers of viral foci within *H. virescens* larval tissues at various h p.i. with either 20 occlusions of AcMNPV-*hsp70/lacZ* or 15 occlusions of HzSNPV-*hsp70/lacZ*. Each point represents the mean number of foci from LacZ-positive larvae in a cohort of between 26 and 33 larvae. Regression lines were fitted by the method of least squares.



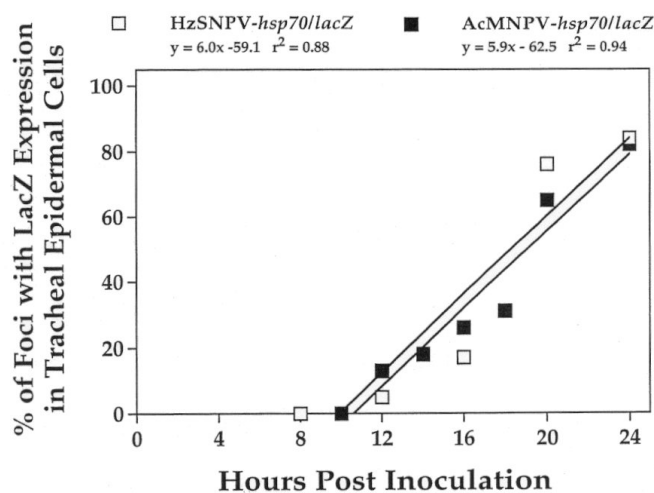
**Fig. 5.** Correlations between the cross-sectional area of HzSNPV-*hsp70/lacZ* and AcMNPV-*hsp70/lacZ* occlusions and number of ODV. Cross-sectional area is presented as weight of paper images of occlusions as described in Methods. Regression lines were fitted by the method of least squares.

Careful determination of the cellular composition of viral foci also revealed that *H. virescens* larvae had sloughed midgut cells infected by HzSNPV-*hsp70/lacZ* and AcMNPV-*hsp70/lacZ* during the 24 h period after inoculation of occlusions (Fig. 6). We found foci with *lacZ* expression restricted to the tracheal epidermal cells, demonstrating that the underlying primary targets had been sloughed previously from the midgut epithelium (Washburn *et al.*, 1998). During microscopic examination of the midgut epithelial tissue from many of these larvae, we also observed round, LacZ-positive columnar cells that were nested within the brush-border membrane and connected to the basal lamina solely by a long, thin strand of cytoplasm; these cells appeared to be in the process of being sloughed from the tissue. Similar proportions of AcMNPV-*hsp70/lacZ* and HzSNPV-*hsp70/lacZ* foci lacked underlying LacZ-positive midgut cells at 16 h p.i., but at 20 and 24 h p.i. there were twofold more such foci in larvae inoculated with AcMNPV-*hsp70/lacZ*. This finding suggested that host larvae sloughed columnar cells infected by the MNPV at greater rates than those infected by the SNPV.

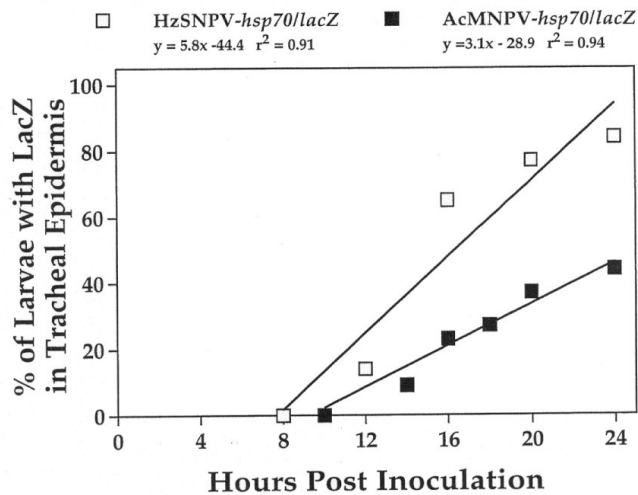


**Fig. 6.** Cellular composition of viral foci in *H. virescens* larvae 16, 20 and 24 h p.i. with either 20 occlusions of AcMNPV-*hsp70/lacZ* (left) or 15 occlusions of HzSNPV-*hsp70/lacZ* (right). Each bar represents the composition of foci in LacZ-positive larvae from a cohort of between 26 and 33 larvae. Numbers above the bars are the numbers of foci characterized at each time-point.

The kinetics for initiation of secondary infection by AcMNPV-*hsp70/lacZ* and HzSNPV-*hsp70/lacZ* in *H. virescens* larvae are shown in Figs 7 and 8. For primary foci of both AcMNPV and HzSNPV, BV transmission to the tracheal epidermis from infected midgut cells was linear from the initiation of systemic infections at 12 to 24 h p.i. when sampling was curtailed. Moreover, the slopes and intercepts of the regression equations describing the timing for establishment of secondary foci of infection by both viruses were nearly the same (Fig. 7). Thus, despite a 6 h difference in the onset of primary infection, the patterns of BV transmission to tracheal epidermal cells from the ODV-infected midgut cells of both the MNPV and the SNPV were virtually identical (Fig. 7). The cumulative effect of BV transmission on the rate at which AcMNPV-*hsp70/lacZ* and HzSNPV-*hsp70/lacZ* systemically infected larvae is shown in Fig. 8. As with individual foci (Fig. 7), the proportions of larvae supporting systemic infections increased in a linear fashion for both viruses, but at different rates. We found that HzSNPV-*hsp70/lacZ* established systemic infections in larval cohorts at a rate that was almost twice that of AcMNPV-*hsp70/lacZ* (i.e. slopes of 5.8 and 3.1, respectively). This finding is not surprising considering that HzSNPV-*hsp70/lacZ* established significantly more primary foci within the first 24 h p.i. per host larva than AcMNPV-*hsp70/lacZ*, and a larger population of primary foci should facilitate transmission of BV into the haemocoel of the host.



**Fig. 7.** Percentages of viral foci with one or more LacZ-positive tracheal epidermal cells at various times after inoculation with 20 occlusions of AcMNPV-*hsp70/lacZ* or 15 occlusions of HzSNPV-*hsp70/lacZ*. Regression lines were fitted by the method of least squares.



**Fig. 8.** Percentages of *H. virescens* larvae with one or more viral foci containing LacZ-positive tracheal epidermal cells at various times after inoculation with 20 occlusions of AcMNPV-*hsp70/lacZ* or 15 occlusions of HzSNPV-*hsp70/lacZ*. Regression lines were fitted by the method of least squares.

## DISCUSSION

Our study of the progression of baculovirus infection in *H. virescens* larvae confirmed that the primary cellular targets of HzSNPV-*hsp70/lacZ* are midgut columnar cells (Granados, 1978; Granados & Williams, 1986), and that the immediate secondary targets of infection were tracheolar cells servicing the midgut (Washburn *et al.*, 2001). Previously, we showed that these are also the primary and immediate secondary targets of AcMNPV-*hsp70/lacZ* during oral infection of *Trichoplusia ni*, *H. zea*, *H. virescens*, and *Manduca sexta* larvae (Engelhard *et al.*, 1994; Engelhard & Volkman, 1995; Washburn *et al.*, 1995, 1996, 1998, 2000). While the sequence of tissues infected during early pathogenesis was the same for both NPVs, the onset of *lacZ* expression in midgut cells infected by HzSNPV-*hsp70/lacZ* ODV was at 4 h p.i. compared to 10 h p.i. for AcMNPV-*hsp70/lacZ*, a 6 h delay ( Fig. 2). Within the lumen of the host's midgut, ODV release from occlusions is virtually instantaneous (J. O. Washburn, unpublished data); hence, the delay in LacZ signalling by AcMNPV-*hsp70/lacZ*-infected midgut cells could be a function of one or more downstream events (e.g. slower nucleocapsid passage within the primary target cells, longer time between nucleocapsid entry into the nucleus and/or onset of viral gene expression). In *H. virescens* haemocytes infected by BV *in vitro*, a delay in *lacZ* expression was observed also, but it was much smaller in comparison, and there were no measurable differences in the replication kinetics of the two genetically diverse recombinants.

Compared to AcMNPV-*hsp70/lacZ*, fewer HzSNPV-*hsp70/lacZ* occlusions were required to achieve the same mortality level in *H. virescens* larvae, and those fewer occlusions generated more than twice the number of primary foci. This result indicated that on a per occlusion basis (the natural unit of infection), the SNPV was the more efficient of the two, but on a per virion basis, the MNPV was more efficient. Previously, we showed that AcMNPV-*hsp70/lacZ* ODV particles containing multiple nucleocapsids were more efficient than AcMNPV-*hsp70/lacZ* ODV containing a single nucleocapsid in establishing mortal

infection in orally inoculated *T. ni* (Washburn *et al.*, 1999). The higher numbers of foci of HzSNPV-*hsp70/lacZ* were correlated with more virions per occlusion relative to AcMNPV-*hsp70/lacZ* (Fig. 5), but other factors such as a greater efficiency of establishing and/or maintaining primary infections by the SNPV may also modulate primary numbers of foci.

In our time-course experiment, despite the 6 h difference in the onset of primary infections, the onset and rate for secondary infection of tracheolar cells were virtually identical (Fig. 7). We observed an 8 h delay between midgut and tracheal infection by HzSNPV-*hsp70/lacZ* compared to only a 2 h delay during AcMNPV-*hsp70/lacZ* pathogenesis. By any study published to date, 2 h is much too short a time for complete replication of a baculovirus. Thus, these results are consistent with the hypothesis that the M character of AcMNPV ODV permits repackaging of parental nucleocapsids as BV, facilitating early infection of the tracheal epidermis before *de novo* virus replication within infected midgut cells (Washburn *et al.*, 1999, 2002). Repackaging the nucleocapsids of AcMNPV ODV is made possible by early expression of GP64, a BV envelope protein absent from ODV (Keddie & Volkman, 1985; Volkman, 1986; Blissard & Rohmann, 1989; Jarvis & Garcia, 1994) but essential for establishment of system infections in *T. ni* and *H. virescens* infected *per os* (Monsma *et al.*, 1996; J. O. Washburn, unpublished data). GP64 is widely conserved among the group I MNPVs (reviewed by Garrity *et al.*, 1997) and is unusual among viral proteins in that its synthesis occurs hours before other structural proteins are made. The M character, coupled with early GP64 expression, provides a mechanism whereby AcMNPV BV can be transmitted to tracheal cells before virus replication is complete. In contrast, HzSNPV lacks both GP64 (Chen *et al.*, 2002) and the M character; thus, BV transmission must be delayed until virus replication occurs in the primary target.

By 24 h p.i., the proportion of LacZ-positive *H. virescens* larvae challenged with HzSNPV-*hsp70/lacZ* (83 %) was equivalent to the final larval mortality (84 %) and notably, all infected larvae in this cohort supported one or more systemic foci of infection. In contrast, only 57 % of larvae inoculated with AcMNPV-*hsp70/lacZ* were infected at 24 h p.i., a level significantly below the final mortality of 87 %; moreover, in a significant number of these larvae, secondary infections had not been established yet (Fig. 2). Our regression equations describing AcMNPV-*hsp70/lacZ* primary and secondary infection kinetics (Figs 3 and 8) predicted that infection levels should equal the final mortality at 30.4 and 37.4 h p.i., respectively, times that coincide with the onset of the first moult after inoculation. These findings suggest that functional primary infections were established by AcMNPV-*hsp70/lacZ* throughout the fourth instar (Washburn *et al.*, 1995). At 32 h p.i., 20 % of the *H. virescens* cohort inoculated with AcMNPV-*hsp70/lacZ* were quiescent and non-feeding, indicating that they were preparing to moult. At this stage, larvae void the contents of the gut lumen and shed infected midgut cells, thereby eliminating both residual ODV and primary infections (Washburn *et al.*, 1995); thus, levels of secondary infection at this premoult stage are predictive of final mortality levels (Engelhard & Volkman, 1995; Washburn *et al.*, 1995). The

fact that our equations predicted the final mortality level coincident with the premoult stage suggests that our linear models based on the LacZ signal accurately reflect the kinetics of AcMNPV-*hsp70/lacZ*.

The extreme sensitivity of *H. virescens* to BV entry of either NPV into the haemocoel underscores the importance for establishing even a single viral focus within the tracheal epidermis, because doing so ensures a productive infection and death of the host. In order to escape virus death, larvae must slough ODV-infected midgut cells prior to their transmission of BV to the haemocoel. Our experiments showed that some primary cellular targets infected by AcMNPV-*hsp70/lacZ* and HzSNPV-*hsp70/lacZ* were shed from the midgut as early as 16 h p.i., many hours before the onset of moulting. Sloughing of AcMNPV-infected midgut cells has been observed previously and photographed (Fig. 2A) (Keddie *et al.*, 1989) and may be a widespread and effective host defence against midgut-initiated virus infection. In our study, we could identify sloughing only for primary foci that successfully transmitted BV to the tracheal epidermis (i.e. via *lacZ* expression restricted to the tracheal epidermis), and frequency data on these foci suggested that midgut cells infected by AcMNPV-*hsp70/lacZ* ODV were sloughed at higher rates than those infected by HzSNPV-*hsp70/lacZ* (Fig. 6). For HzSNPV-*hsp70/lacZ*, foci numbers rose continuously for 16 h after the onset of primary infection (i.e. 4–20 h p.i.); thus, if infected midgut cells were sloughed prior to 16 h p.i., the rate of loss was insufficient to counter the rise in new primary infections. Moreover, by 16 h p.i., 89 % of the LacZ-positive larvae (65 % of the cohort; Fig. 2) inoculated with HzSNPV-*hsp70/lacZ* already supported secondary tracheal foci, indicating that subsequent sloughing would not have cleared infections. Throughout primary infection, numbers of AcMNPV-*hsp70/lacZ* foci remained substantially below those achieved by HzSNPV-*hsp70/lacZ* and reached a maximum at 16 h p.i., only 6 h after the onset of primary infection. We showed previously that when *H. virescens* larvae were orally inoculated progressively later during the fourth instar, AcMNPV-*hsp70/lacZ* exhibited a concomitant decreasing ability to establish and/or maintain primary infection (Washburn *et al.*, 1998). The delay in the onset of AcMNPV-*hsp70/lacZ* primary infection, the decline in numbers of primary foci after 16 h p.i. and the higher frequency of foci lacking primary infections provide evidence that the cellular targets infected by the MNPV were sloughed more frequently than those infected by the SNPV. This effect was counteracted, however, by the shorter time-lag between primary and secondary AcMNPV-*hsp70/lacZ* infection.

In summary, we have shown that both AcMNPV-*hsp70/lacZ* and HzSNPV-*hsp70/lacZ* transiently infect midgut columnar cells, which subsequently transmit BV to the host's haemocoel by infecting tracheolar cells. *H. virescens* larvae sloughed primary cellular targets of both NPVs, and the two pathogens countered this host defence in different ways. HzSNPV-*hsp70/lacZ* initiated primary infections more quickly and in greater numbers than AcMNPV-*hsp70/lacZ*, but secondary infection by the SNPV was delayed by 8 h from the onset of primary infection. In contrast, AcMNPV-*hsp70/lacZ* established secondary infection only 2 h after establishing primary infections and continued to establish primary

infections late into the instar. The difference in time intervals between primary and secondary infections support the hypothesis that the earliest secondary targets of the SNPV were infected by newly synthesized BV, whereas those of the MNPV were infected by parental ODV nucleocapsids repackaged as BV. Remarkably, BV transmission by both viruses to secondary target cells occurred at the same rate and time, suggesting that the infection strategies of both HzSNPV and AcMNPV have been selected for rapid establishment of systemic infection. To test directly if the earliest tracheal infections by AcMNPV are established by repackaged ODV nucleocapsids, we plan to label nucleocapsids radioactively and monitor their distribution in larval tissues.

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