Lack of involvement of haemocytes in the establishment and spread of infection in *Spodoptera frugiperda* larvae infected with the baculovirus *Autographa californica* M nucleopolyhedrovirus by intrahaemocoelic injection

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It is thought that insect haemocytes, or blood cells, play an important role in baculovirus pathogenesis by amplifying and helping to spread the infection within the insect. Here, infection is described of the lepidopteran noctuid species *Spodoptera frugiperda* with the baculovirus *Autographa californica* M nucleopolyhedrovirus (AcMNPV). Late instar *S. frugiperda* larvae were infected by intrahaemocoelic injection using a recombinant of AcMNPV expressing the enhanced green fluorescent protein gene to visualize infected cells. Approximately 1000-fold higher doses of injected virus were required to initiate infection in *S. frugiperda* larvae than in another permissive noctuid species, *Trichoplusia ni*. Infected *S. frugiperda* larvae survived twice as long as *T. ni* larvae and exhibited a slower build-up of virus in the haemolymph. In *S. frugiperda*, infection of fat body and epithelium was observed prior to significant infection of haemocytes, even though the virus was delivered by intrahaemocoelic injection. Expression of eGFP was first detected 12–18 h post-injection within the fat body and, by 24 h, infection had spread to the tracheal and body wall epithelium. In contrast, only 5% of haemocytes were infected at 24 h and the proportion of infected haemocytes increased slowly to only around 50% at 5 days post-infection, when most larval death occurred. Thus, in *S. frugiperda*, haemocytes do not appear to have a primary role in AcMNPV pathogenesis. This relative lack of infection of haemocytes may in part explain why *S. frugiperda* larvae are more resistant to AcMNPV infection than *T. ni* larvae.

Introduction

Baculoviruses are rod-shaped, enveloped, double-stranded DNA viruses that specifically infect arthropods, with most of the several hundred baculoviruses that have been described infecting members of the order Lepidoptera. Although the majority of baculoviruses are limited to a few closely related host caterpillars, the type species of the *Nucleopolyhedrovirus* genus, *Autographa californica* M nucleopolyhedrovirus (AcMNPV), has a host range encompassing over a dozen families of Lepidoptera, including a number of important pest species (Granados & Williams, 1986). AcMNPV occurs in two different forms – as enveloped clusters of virions packaged in a proteinaceous matrix of polyhedrin (occluded virus) or as single-enveloped virions (budded virus). The natural cycle of infection begins when occluded virus is ingested by feeding caterpillars. The polyhedrin matrix of the occlusion bodies dissolves rapidly in the alkaline conditions of the caterpillar midgut, releasing the virions that cross the peritrophic membrane and infect the cells of the midgut epithelium. The infection is thought to spread from the midgut across the basal lamina and into the body of the caterpillar via the tracheoblast cells (Engelhard *et al*., 1994). Once the virus has entered the body of the caterpillar, virus infection occurs simultaneously within the haemocoel and the tracheal system of the host insect and spreads rapidly to the epithelial and fat body tissues (Keddie *et al*., 1989; Engelhard *et al*., 1994; Flipsen *et al*., 1995). Eventually, nearly all tissues become infected and death of the caterpillar is accompanied by ‘melting’ or liquefaction of the...
tissues. In addition to feeding on occluded virus, larvae can also be infected experimentally by intrahaemocoelic injection with budded virus. Although less well characterized, at a gross level, the pathology of such infections is similar to that seen in infections initiated by feeding; infections performed by the injection route can yield useful information concerning the progression of infection once the virus has penetrated the midgut basal lamina and entered the body proper of the caterpillar.

The progress of AcMNPV infection within the host has been best described for the cabbage looper caterpillar Trichoplusia ni. These T. ni caterpillars are extremely susceptible to AcMNPV infection, with the injection LD$_{50}$ (dose required for 50% lethality) being reported as between 1 and 10 p.f.u. per larva (Clem & Miller, 1993) or less (Washburn et al., 1998) and a feeding LD$_{50}$ of 15 occlusion bodies per larva (Washburn et al., 1998). Virus can be observed in the haemolymph of the infected insect within 10 h following ingestion of occluded virus and blood titres rise rapidly over the first 24 h of infection before reaching a plateau (Granados & Lawler, 1981). Infection of haemocytes and trachea can be detected 12–24 h after oral infection, followed by the spread of the virus to the epithelial and fat body tissues (Barrett et al., 1998; Keddie et al., 1989). Death of the caterpillar occurs four days after the initial infection and the time to death is only slightly shortened if the virus is delivered by injection (Washburn et al., 1995). Studies of developmental and selected resistance in a number of susceptible caterpillar species suggest that the sloughing of infected midgut cells is the primary defence against baculovirus infection and that few insects are capable of resisting the virus once it crosses the midgut basal lamina (Volkman, 1997; Washburn et al., 1998).

The AcMNPV infection process has been examined in detail in only a handful of other lepidopteran species, including the tobacco budworm (Heliothis virescens), corn earworm (Helicoverpa zea) and armyworm (Pseudaelia unipuncta). The infection process of AcMNPV in H. virescens follows a pattern similar to that in T. ni, with a longer period between infection of midgut cells and movement of the virus across the basal lamina and a slightly slower progression of the virus through the insect tissues (Washburn et al., 1995). Injection of AcMNPV into P. unipuncta also produces an infection pattern resembling that of T. ni; however, this species is highly resistant to oral infection (Volkman, 1997). Unlike the aforementioned species, H. zea is able to limit the progression of the virus after it has crossed the midgut by encapsulating the infected tracheal cells with haemocytes and by using the haemocytes, which appear to be resistant to infection, to clear virions from the blood (Washburn et al., 1996; Trudeau et al., 2001).

In the course of examining the role of apoptosis in insect defence, we noted that fall armyworm (Spodoptera frugiperda) caterpillars undergo a somewhat different response than T. ni to infection of AcMNPV budded virus. In this study, we used a recombinant strain of AcMNPV expressing enhanced green fluorescent protein (eGFP) to document the course of infection in the haemolymph, epidermal and fat body tissues of S. frugiperda and to examine the role of haemocytes in the dissemination of baculovirus infection following intra-haemocoelic injection.

**Methods**

- **Virus construction.** The recombinant virus vHSGFP was constructed by inserting a cassette containing the eGFP gene under the control of the Drosophila hsp70 heat shock promoter into the L1 strain of AcMNPV at a site adjacent to the polyhedrin gene (Fig. 1). DNA extracted from the occlusion-negative, lacZ-expressing recombinant virus vSyMvGal (Wang et al., 1991) was cotransfected with the plasmid pHS70GFPBu316 (containing eGFP under hsp70 promoter control) into TN-368 cells using lipid-mediated transfection. Infected cells were maintained in TC-100 media (Invitrogen) containing 10% foetal bovine serum (FBS) (BioWhittaker or Invitrogen) and the virus-containing supernatant was collected after 48 h. Three rounds of plaque purification were used to isolate an eGFP-positive, occlusion-positive, lacZ-negative plaque that was used for the subsequent generation of high-titre vHSGFP. Virus stocks were grown and titrated on TN-368 cells by plaque assay, as described previously (O’Reilly et al., 1992). Plaques were enumerated at 3 days post-infection by eGFP fluorescence.

- **Caterpillars.** T. ni eggs were obtained from Entopath (Easton, PA, USA), while S. frugiperda eggs were obtained from Agrilept (Zebulon, NC, USA). Individual first instar caterpillars were transferred into 1 oz cups containing blocks of either cabbage looper or fall armyworm diet, respectively, and reared at 27 °C with a 12 h light–dark cycle. Both diets were obtained from Southlands Products (Lake Village, AR, USA) and made according to the manufacturer’s specifications.

- **Injections.** T. ni and S. frugiperda caterpillars were injected with measured doses of budded virus diluted in complete TC-100 media within 12 h of molting to the penultimate (S. frugiperda) or final (T. ni) instar. T. ni caterpillars were injected in the final instar to reduce injection-related mortality, which was less than 1% in each species. Intrahaemocoelic injections were performed with a Hamilton 26 gauge syringe by inserting the needle between the first and second abdominal segment of the caterpillar and depositing the virus at the rear of the abdomen.

- **Haemolymph extraction and examination.** For each experiment, cohorts of 10 caterpillars were examined every 24 h for external eGFP fluorescence and then sacrificed. Caterpillars were chilled for 30 min at 4 °C before haemolymph extraction. Haemolymph was collected by cutting an anal proleg and allowing the caterpillar to bleed on to a section of parafilm. Of the haemolymph, 5 μl was then transferred into 200 μl of ice-cold anti-coagulant buffer (4 mM NaCl, 40 mM KCl, 1.7 mM PIPES, 146 mM sucrose, 0.1% polyvinyl pyrrolidone, 8 mM EDTA, 9.5 mM citric acid and 27 mM sodium citrate). Samples were centrifuged at 320 r.p.m. and 4 °C to separate the cells from the haemolymph. Cells were fixed in 2% paraformaldehyde and examined on a Nikon Eclipse TE200 inverted microscope for the presence of eGFP fluorescence. A minimum of 100 cells was scored from each sample. Cell-free haemolymph samples were stored at −80 °C for determination of virus levels. Inspection of infected caterpillars was performed using a Nikon SMZ-10A binocular microscope equipped for eGFP fluorescence. Internal examinations were performed on a subset of each cohort of caterpillars to confirm infection status. Fat body was removed and viewed using the inverted microscope described above.
were performed on each cohort of caterpillars prior to haemolymph extraction and the degree of infection was assessed based on the degree of eGFP fluorescence.

* T. ni* caterpillars injected with $4 \times 10^8$ p.f.u. of vHSGFP displayed strong overall external eGFP fluorescence and strong internal fluorescence in the epithelial, fat body and tracheal tissue at 24 h after injection (data not shown). * T. ni* caterpillars injected with 40 p.f.u. of vHSGFP showed a reduced level of infection 24 h after injection, with most insects displaying scattered spots of infection internally and externally throughout the body but with a minority (6 of 20 caterpillars) having no external eGFP expression and no obvious internal sign of infection. However, by 48 h post-injection, all caterpillars receiving 40 p.f.u. of virus exhibited strong eGFP expression both internally and externally (data not shown).

* T. ni* injected with either 40 or $4 \times 10^8$ p.f.u. of vHSGFP showed nearly simultaneous expression of eGFP in epithelia, tracheal epithelia, haemocytes and fat body by 18 h post-injection. The majority of initial foci of infection in the fat body occurred in close proximity to the trachea but without extensive infection of the tracheal epithelium. Externally, foci initially appeared to be randomly distributed throughout the epithelia but, by 24 h, infection was strongest in the thoracic legs, prolegs and the mid-region of each abdominal segment.

At 24 h after injection, 30% of the * S. frugiperda* caterpillars receiving $2 \times 10^5$ p.f.u. of virus showed significant external infections, with large numbers of scattered foci consisting of one or a few cells (Table 1 and Fig. 3). The remaining caterpillars either had no obvious external sign of infection or had only one or a few isolated foci of infection. The majority of the initial foci of infection were concentrated in the thoracic legs and ventral abdomen during the first 48 h after injection, with little eGFP expression occurring dorsally until the infection had become well established. Once the infection had become established, eGFP expression increased rapidly throughout the insect until by 72 h, 90% of the caterpillars displayed solid eGFP expression throughout the epithelium and fat body (Table 1). Caterpillars injected with $2 \times 10^4$ p.f.u. showed little or no infection 24 h after injection but, by 48 h, 50% of the caterpillars displayed multiple foci of infection and, by 96 h, 85% of the caterpillars were showing solid eGFP expression throughout their tissues (Table 1).

At 12–18 h after injection, internal inspection of * S. frugiperda* caterpillars injected with $2 \times 10^8$ p.f.u. of vHSGFP revealed between 20 and 50 small foci of infected cells randomly distributed throughout the fat body of the insect. As with * T. ni*, the majority of the foci were located well within the fat body and did not abut the basal lamina and most were within a few cell widths of a tracheal tube (Fig. 3E, F). In a very small percentage of * S. frugiperda* caterpillars, some of the eGFP expression within the fat body could be directly attributed to infection that occurred during the injection process; however, even within these caterpillars, the majority of the eGFP-positive foci were located away from the wound site, indicating...
that the virus had become widely distributed throughout the caterpillar following injection.

In contrast to infection in *T. ni* where eGFP expression appeared almost simultaneously in a number of different tissues, eGFP expression in *S. frugiperda* was not detected outside of the fat body between 12 and 18 h after injection. However, by 18–24 h, eGFP expression could be observed externally within the body wall of infected *S. frugiperda* and internally in the tissues surrounding the spiracle entrances. By this time, infection within the fat body had spread from the initial foci to form web-like patterns of eGFP expression. eGFP-positive tracheal epithelial cells could also be observed at 18–24 h after injection.

**Haemolymph virus levels and haemocyte infection**

*T. ni* caterpillars injected with $4 \times 10^3$ p.f.u. of vHSGFP exhibited a rapid build-up of virus in the haemolymph by 24 h after injection, with virus levels remaining high throughout the course of the infection (Fig. 4A). Similarly, by 24 h, the proportion of eGFP-positive haemocytes in these caterpillars had reached 57% and, by 48 h, eGFP could be detected in 90% of haemocytes (Fig. 5A). *T. ni* caterpillars injected with 40 p.f.u. of vHSGFP displayed a slower initial build-up of infection. At the 24 h time-point, the proportion of eGFP-positive haemocytes in *T. ni* caterpillars injected with 40 p.f.u. was significantly lower than in caterpillars injected with $4 \times 10^5$ p.f.u. (Fig. 5A). The levels of virus among these caterpillars were high 24 h after injection but displayed a lower mean and a greater degree of variation than the levels of virus in caterpillars injected with $4 \times 10^3$ p.f.u. (Fig. 4A). However, by 48 h, these caterpillars had similarly high levels of eGFP-positive haemocytes and haemolymph virus levels.

In contrast, *S. frugiperda* caterpillars injected with $2 \times 10^5$ p.f.u. of vHSGFP showed a slow build-up of infection in the haemolymph despite the rapid spread of infection through the epidermal, fat body, and tracheal tissues (Figs 4B and 5B). The amount of virus in the haemolymph of these caterpillars

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**Table 1.** Proportions of *S. frugiperda* caterpillars exhibiting different degrees of external eGFP expression following injection with vHSGFP

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**Fig. 2.** Mortality of *S. frugiperda* and *T. ni* caterpillars due to infection by vHSGFP. A total of 20 larvae was injected per dose and the per cent mortality calculated by dividing the number of larvae that succumbed to virus infection (as determined by eGFP expression and general appearance) by the number of larvae injected (less any larvae that died from causes unrelated to the infection).
increased over the first 72 h after injection before reaching a plateau (Fig. 4B). At 120 h, the caterpillars started to die and, by 144 h, the majority of the caterpillars were dead, preventing further samples from being taken. The proportion of eGFP-positive haemocytes in these caterpillars showed a slow but steady increase throughout the course of infection, from a mean of less than 5% eGFP-positive haemocytes at 24 h to a mean of 52% at 120 h post-injection (Fig. 5B). Although the caterpillars that received 2 × 10⁴ p.f.u. of virus had very little detectable virus in their haemolymph at 24 h post-injection, the virus increased in the haemolymph at a similar rate as the caterpillars receiving 2 × 10³ p.f.u. and reaches its highest level 120 h after infection (Fig. 4B). The proportion and rate of increase in eGFP-positive haemocytes from caterpillars injected with 2 × 10³ p.f.u. of vHSGFP did not differ significantly from that of caterpillars injected with 2 × 10⁴ p.f.u. (Fig. 5B).

Discussion

The pattern of AcMNPV infection observed in the caterpillar S. frugiperda shows a number of significant differences from that of T. ni and other similarly susceptible caterpillar species. T. ni larvae are extremely susceptible to AcMNPV infection, requiring an injection of as little as 1 p.f.u. per caterpillar to induce 95% mortality. Infected T. ni caterpillars rapidly built up high levels of virus in the haemolymph within the first 24 h after injection and exhibited a rapid increase in the proportion of infected haemocytes, until, by 48 h post-injection, 90% of the haemocytes exhibited eGFP expression and the virus had spread to all susceptible tissues within the insect. By 72 h post-injection, the first deaths were...
observed among the infected caterpillars and, by 96 h, all caterpillars had died. *T. ni* larvae have also been shown to be highly susceptible to infection by oral feeding of AcMNPV polyhedra (Keddie et al., 1989), oral feeding of infected insect cadavers (Barrett et al., 1998), direct injection of tracheoblasts via the trachea (Kirkpatrick et al., 1994) and infection via intrahaemocoelic injection (Clem & Miller, 1993; Danyluk & Maruniak, 1987). Oral infection of *T. ni* caterpillars with occluded virus or budded virus from insect cadavers results in an internal infection within 12–30 h, with virus first appearing in the midgut connective tissue, haemocytes and tracheal epithelia before spreading into the epidermis and fat body, killing the insect within 72–96 h.

*S. frugiperda*, in contrast, is less susceptible to AcMNPV. The dose of virus required to kill 50 and 95% of the *S. frugiperda* caterpillars was in each case 1000 times greater than the dose required to kill an equal proportion of *T. ni* caterpillars. *S. frugiperda* caterpillars injected with vHSGFP showed a slower increase in the amount of budded virus in the haemolymph than *T. ni* caterpillars. Expression of eGFP was also slow to appear in the haemocytes of infected *S. frugiperda* caterpillars and significant infection of haemocytes was not observed until fairly late in the infection process. Although the proportion of infected haemocytes increased steadily throughout the course of the infection, by 120 h post-injection, at which time the *S. frugiperda* larvae were beginning to die, the proportion of eGFP-positive haemocytes was still significantly less than the proportion of infected haemocytes observed in *T. ni* at 48 h post-injection. Despite the slow build-up of virus in the haemolymph of *S. frugiperda*, the infection managed to spread into the epidermal and fat body tissues of *S. frugiperda* caterpillars relatively rapidly, although the rate at which these tissues were infected was dependent upon the dose of the initial inoculum.

Another significant difference we observed between the two species was the order in which tissues became infected. In *T. ni*, eGFP expression appeared simultaneously in a number of different susceptible tissues, including fat body, tracheal and body wall epithelium and haemocytes at around 12–18 h post-injection. However, in *S. frugiperda*, it was clear that the fat body was the tissue where infection first occurred. Infection appeared to spread from the fat body to tracheal and body wall epithelium by 18–24 h post-injection but significant numbers of haemocytes did not become infected until much later.

Earlier studies of the pathology of AcMNPV in *T. ni* and *H. virescens* have involved either oral or tracheal inoculation and have reported a steady progression of infection from the midgut into the trachea and haemocoel and from there into the epidermis and fat body. In our examination of the pathology of AcMNPV in *S. frugiperda*, we observed that the initial foci of infection occurred well within the fat body of the caterpillars before infection could be detected in the trachea, haemocytes or body wall. The mechanism by which the virus penetrated the fat body is unclear; however, the close proximity of trachea to many of the foci of infection suggest that the virus may be able to utilize the tracheoblast cells as a conduit to travel into and infect susceptible tissues. The lack of eGFP expression within the tracheal system at this early time indicates that such a transportation mechanism would have to occur without infecting the tracheoblast cells or by using tracheoblast cells that are infected but in which viral DNA is not being transcribed, since the heat shock promoter used to drive eGFP expression in this study is expressed constitutively in most, if not all, cell types. The process of using infected tracheoblast cells to rapidly transport other virions deeper into the host tissues has been suggested as a mechanism by which MNPV baculoviruses are able to rapidly cross the midgut basal lamina before the midgut epithelium is sloughed off (Grandos, 1978; Granados & Lawler, 1981; Washburn et al., 1999) and...
enter other organs in *T. ni* (Engelhard et al., 1994). A similar mechanism may be involved during the infection of *S. frugiperda*, allowing the virus to rapidly penetrate deep into the tissues of its host during the very early stages of the infection.

In general, the mechanisms by which a caterpillar could resist baculovirus injected into its body fall into two basic classes: those that operate at the level of the infected cell and those that operate at the level of the organism. The large difference in susceptibility to AcMNPV infection between *T. ni* caterpillars and *S. frugiperda* and the slower progression of infection in the latter species strongly suggests that *S. frugiperda* possesses a partially effective mechanism of virus resistance, capable of preventing infection from low doses of virus but incapable of stopping infection once the virus has become established. In particular, the observation that injecting 2 × 10³ p.f.u. of virus into *S. frugiperda* caterpillars is sufficient to initiate only 30–50 initial foci of infection indicates that either large quantities of virus are inactivated and/or removed from the haemolymph following infection or that *S. frugiperda* cells are much more resistant to AcMNPV infection than are *T. ni* cells. Stocks of AcMNPV titrated on *S. frugiperda* cell lines produce a virus titre approximately 40 times lower than when titrated on *T. ni* cells (Danyluk & Maruniak, 1987), an observation that suggests that a ‘cellular-level’ resistance mechanism may be at least partially responsible for the difference in susceptibility between these two caterpillar species. However, of the cellular mechanisms of resistance to baculovirus infection that have been described, including apoptosis (Clem & Miller, 1993), protein synthesis shutdown (Du & Thiem, 1997) and incompatibility between virus replication machinery and the host cell (Crozier et al., 1994), none appear to be involved in limiting the ability of *S. frugiperda* caterpillars to be infected with AcMNPV.

‘Organism-level’ resistance mechanisms that would block infection by injected virus have been described for the species *H. zea* (Trudeau et al., 2001) but there is little indication that similar mechanisms are functioning within *S. frugiperda*. *S. frugiperda* haemocytes do not form melanotic capsules around infected tissue and, unlike *H. zea* haemocytes, a large proportion of the *S. frugiperda* haemocytes do eventually become infected. The more gradual increase in the proportion of infected haemocytes in *S. frugiperda* relative to *T. ni* suggests that these cells may have a role in resisting infection and removing virus from the blood, similar to the situation in *H. zea* (Trudeau et al., 2001). However, alternative explanations, such as rapid replacement of infected haemocytes, removal of infected haemocytes by phagocytic tissue or slower up-take of virus by the haemocytes cannot be ruled out.

In summary, we have provided evidence that the establishment of systemic infection by AcMNPV in *S. frugiperda* does not require significant infection of haemocytes, despite the injection of large amounts of budded virus directly into the haemolymph. Instead, the first tissue infected is the fat body, followed by epidermal cells lining the trachea and body wall. Around 50% of the haemocytes eventually become infected but most do not become infected until very late in the infection process after the virus has already spread to other tissues. In this study, we infected caterpillars by intraheemocoelic injection in order to examine virus pathogenesis and organism-level resistance without the complications of the midgut barrier. In a natural setting, caterpillars would be infected by feeding on occluded virus particles. It will thus be of interest to compare these results with the pattern of infection that is seen following the establishment of infection by feeding of occluded virus.

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**References**


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