Response of immunocompetent and immunosuppressed Spodoptera littoralis larvae to baculovirus infection

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The Mediterranean lepidopteran pest Spodoptera littoralis is highly resistant to infection with the Autographa californica multiple nucleopolyhedrovirus (AcMNPV) via the oral route, but highly sensitive to infection with budded virus (BV) via the intrahaemocoelic route. To study the fate of AcMNPV infection in S. littoralis, vHSGFP, an AcMNPV recombinant that expresses the reporter green fluorescent protein gene under the control of the Drosophila heat-shock promoter, and high-resolution fluorescence microscopy were utilized. S. littoralis fourth-instar larvae infected orally with vHSGFP showed melanization and encapsulation of virus-infected tracheoblast cells serving the midgut columnar cells. At 72 h post-infection, the viral foci were removed during the moult clearing the infection. Thus, oral infection was restricted by immune responses to the midgut and midgut-associated tracheal cells. By contrast, injection of BV into the haemocoel resulted in successful infection of tracheoblasts, followed by spread of the virus through the tracheal epidermis to other tissues. However, in contrast to fully permissive infections where tracheoblasts and haemocytes are equally susceptible to infection, a severe limitation to vHSGFP infection of haemocytes was observed. To investigate the resistance of S. littoralis haemocytes to BV infection with AcMNPV, the larval immune system was suppressed with the Chelonus inanitus polydnavirus or a putatively immunosuppressive polydnavirus gene, P-vank-1. Both treatments increased the susceptibility of S. littoralis larvae to AcMNPV. It is concluded that the resistance of S. littoralis to AcMNPV infection involves both humoral and cellular immune responses that act at the gut and haemocyte levels. The results also support the hypothesis that tracheolar cells mediate establishment of systemic baculovirus infections in lepidopteran larvae. The finding that polydnaviruses and their encoded genes synergize baculovirus infection also provides an approach to dissecting the responses of the lepidopteran immune system to viruses by using specific polydnavirus immunosuppressive genes.

INTRODUCTION

The factors involved in determination of host range of viruses in the family Baculoviridae, which infect arthropods (mainly insects), are poorly understood (Ayres et al., 1994; Lange et al., 2004). To investigate determinants of the baculovirus host range, we developed a model system based on infection of the Mediterranean pest Spodoptera littoralis (Chejanovsky & Gershburg, 1995; Gershburg et al., 1997; Lu et al., 2003) with the prototype of the genus Nucleopolyhedrovirus, the Autographa californica multiple nucleopolyhedrovirus (AcMNPV). The genome of AcMNPV has been sequenced completely (Ayres et al., 1994), facilitating the identification and study of the viral genes involved in the infection process as well as the design of recombinant baculoviruses (Possee, 1997). S. littoralis larvae are highly resistant to oral infection (via viral polyhedra, PIB) by AcMNPV (Bishop et al., 1988). We have shown previously that AcMNPV induces apoptosis of S. littoralis SL2 cells (Chejanovsky & Gershburg, 1995) due to poor expression of the apoptotic suppressor p35, in contrast to Spodoptera littoralis nucleopolyhedrovirus (SpliNPV)-infected cells (Du et al., 1999). Further, we demonstrated that recombinant AcMNPV lacking or poorly expressing the immediate-early gene ie0 replicated in SL2 cells to yield higher budded virus (BV) titres than the wild-type virus and displayed improved infectivity upon intrahaemocoelic injection of S. littoralis larvae (Lu et al., 2003, 2005). However, these studies did not
result in significant enhancement of the oral infectivity of these AcMNPV recombinants towards S. littoralis larvae, suggesting that other host-related factors, such as an immune response, suppress the viral infection (Lu et al., 2003).

Polydnaviruses (PDVs) have segmented, double-stranded DNA genomes and obligate mutualistic associations with some parasitic hymenoptera (families Ichneumonidae and Braconidae). PDVs suppress the immune responses of parasitized lepidopteran insects to the wasp egg and larvae, whilst the wasp ensures the propagation and dispersal of the viral genome (Fleming, 1992; Kroemer & Webb, 2004). During oviposition, the PDV is introduced with the parasite egg, venom and ovarian proteins (OPs) from the female reproductive tract into parasitized larvae [such as Campopleis sonorensis ichnovirus (CsIV), ichnovirus group] or eggs [like Chelonus inanitus bracovirus (CiBV), brachovirus group]. In the lepidopteran host, PDVs infect host cells (haemocytes, muscle, fat body and malpighian tubules) (Stoltz & Vinson, 1979), but do not replicate, although expression of a host-specific subset of viral genes is detectable within a few hours of parasitization (reviewed by Kroemer & Webb, 2004). Chelonus inanitus is a braconid, endoparasitic wasp that bears CiBV. Parasitation of S. littoralis by Chelonus inanitus involves immunosuppression by oviposition of the wasp eggs into S. littoralis eggs. The PDV is introduced into S. littoralis eggs during oviposition as a component of the calyx fluid that surrounds wasp eggs in the oviduct (Wyder et al., 2003). The CiBV genes responsible for host immunosuppression have not been identified, so it is used in this study as a means to broadly suppress host immune responses.

The recently completed genome sequence of the CsIV genome and identification of a gene family, the vankyrin gene family, thought to interfere with immune responses provide a specific and complementary approach to investigating host immune suppression (Kroemer & Webb, 2004). The viral vankyrins have ankyrin-repeat domains similar to the Drosophila Dorsal/NF-κB transcription-factor inhibitor cactus (Kroemer & Webb, 2004). However, this viral family of genes encodes only the inhibitory ankyrin-repeat domains and lacks the regulatory domains found on cactus and other cellular IκBα. Our hypothesis is that these genes function as irreversible inhibitors that block immune-cell signal transduction and thereby prevent effective cellular immune responses of the host (Kroemer & Webb, 2004).

To study the fate of the AcMNPV infection in the S. littoralis host and the ability of the host to mount an immune response against this baculovirus, we utilized vHSGFP, an AcMNPV recombinant that expresses the reporter green fluorescent protein (GFP) gene under control of the Drosophila heat-shock promoter that is activated constitutively in insect cells (Clarke & Clem, 2002) and high-resolution fluorescence microscopy. Further, we assessed the effect of general and specific PDV-mediated immunosuppression of S. littoralis on the infectivity of AcMNPV towards these larvae.

**METHODS**

**Cell lines and viruses.** Spodoptera frugiperda Sf9 cells were maintained and propagated in TNM-FH medium supplemented with 10 % heat-inactivated fetal bovine serum (Summers & Smith, 1978). Wild-type AcMNPV E-2 strain (Smith & Summers, 1978; Summers & Smith, 1978) and vHSGFP (Clarke & Clem, 2002; Summers & Smith, 1978) were propagated on Sf9 cells. Viral growth curves were determined by plaque assay as described previously (Du et al., 1999).

**Bioassays.** Carefully selected fourth-instar S. littoralis larvae (25 per dose, triplicates) were injected with 1 μl of various doses from 10 to 10⁵ BV of AcMNPV or vHSGFP. Control larvae were injected with the same volume of TNM-FH complete medium. Percentage mortality was calculated as the number of dead larvae divided by the total number of larvae (excluding larvae killed by the injection, normally one or two) × 100. No mortality was observed for mock-infected larvae (Chejanovsky & Gershburg, 1995; Chejanovsky et al., 1995; Gershburg et al., 1997).

Oral-infection bioassay was performed by inoculating recently eclosed fourth-instar S. littoralis larvae with 1 μl medium containing doses of vHSGFP ranging from 500 to 100,000 PIBs directly into the mouth and anterior of the midgut by using a micromanipulator. After 24 h exposure to the virus, larvae were transferred to new containers containing non-contaminated diet and larval death was monitored daily for 3 weeks. Twenty-five larvae were used for each dose and the experiment was repeated twice.

**Chelonus inanitus parasitization of S. littoralis.** S. littoralis eggs were exposed to Chelonus inanitus 25–32 h after oviposition following published methodology (Kaeslin et al., 2005). Parasitization was verified by dissection of a few eggs. S. littoralis eggs were reared and, when the larvae reached the fourth instar, they were injected with various doses of AcMNPV BV or mock-infected as described above. Hatching of Chelonus inanitus larvae of the mock-infected eggs and mock-infected larvae was monitored carefully to ensure the validity of the bioassay.

**Examination of larvae for GFP expression.** Larvae were secured onto wax plates with minutin pins through the head capsule and terminal abdominal segments. The specimens were overlaid with CEB buffer: 10 mM PIPES (pH 6.8), 60 mM sucrose, 100 mM KCl, 5 mM magnesium acetate, 1 mM EGTA (Engelhardt et al., 1994) and a single, longitudinal incision was made through the dorsal cuticle. Insects inoculated with PIBs were examined as above after making a longitudinal incision through the midgut. All of the specimens were placed immediately in fixative (2 % p-formaldehyde/CEB) after removal of CEB. GFP expression was monitored by using an Olympus IX 81(Japan) inverted laser-scanning confocal microscope (Fluoview 500) equipped with a 488 nm argon-ion laser and a 60 × 1.0 NA PlanApo water immersion objective. GFP was excited by 488 nm light and the emission was collected through a BA 515–525 filter. Magnifications were performed by zooming the scanning laser beam onto a smaller area of the object. The transmitted light images were obtained by using Nomarski differential interference contrast (DIC) microscopy.

**Haemolymph examination.** Haemocytes were collected by bleeding the larvae at their proleg into anticoagulant buffer: 98 mM NaOH, 186 mM NaCl, 1.7 mM EDTA and 41 mM citric acid buffer, pH 4.5 (Strand & Pech, 1995), then observed immediately under the fluorescent microscope.
RESULTS

**S. littoralis resists oral infection with AcMNPV**

Preliminary data indicated that *S. littoralis* larvae were completely resistant to oral infection by ingestion of AcMNPV polyhedra, but susceptible to infection by injection of BV. To study the infection pathways of AcMNPV in the body of *S. littoralis* after ingestion or injection, we utilized a polyhedra-positive AcMNPV recombinant, vHSGFP, that expresses the GFP gene under the control of the *Drosophila* heat-shock promoter. Oral infection was performed by inoculating recently ecdysed fourth-instar *S. littoralis* larvae with doses of vHSGFP ranging from 500 to 100 000 PIBs into the mouth, foregut and anterior midgut by using a micromanipulator to deliver an exact number of polyhedra to the insect (Engelhard *et al*., 1994). No larval mortality was observed after these oral infections (not shown). To investigate the fate of the recombinant virus in the infected larvae, we monitored for signs of infection and reactions of the host to the viral dose. At 6 h post-infection, many GFP-fluorescent viral foci appeared in the lumen of the midgut epithelium (Fig. 1a). At 24 h post-infection, 40–60 % of the virus-infected larvae exhibited melanization proximal to the columnar epithelial cells of the midgut. This corresponded to foci of virus-infected cells expressing GFP (Fig. 1b, c; see below). As expected, no melanization or GFP expression was detected in the midgut of mock-infected larvae (Fig. 1d). At 48 h post-infection, the percentage of larvae showing viral foci and the size of foci decreased by four- to sixfold. At

![Fig. 1. Oral infection of *S. littoralis* larvae with vHSGFP. Midgut mounts of infected larvae (50 000 PIBs). (a) Confocal microscopic view of a vHSGFP-infected midgut showing the infected fluorescent foci. Bar, 50 μm. (b, c) Left panels, binocular picture showing brown patches of melanization in infected larvae; magnification ×80 and ×40, respectively. Right panels, GFP fluorescence. (c) A delicate scraping of the midgut surface, showing the clear correlation between the melanin patches (arrows) and the viral GFP-expressing infected cell. (d) Mock-infected larvae. (e, f) Closer confocal microscopic view of GFP-signalling midgut cells and servicing tracheal cells. Melanization is indicated by arrows. Dark and lower panels, fluorescent and superimposed pictures, respectively. Bars, 5 μm.](http://vir.sgmjournals.org)
72–96 h post-infection, virus-infected cells were not detected and all foci were cleared, probably due to the moult of these larvae to the fifth instar (not shown). Detailed analysis of the fluorescent foci in all larvae examined indicated that vHSGFP infected the midgut epithelial cells and tracheoblast cells servicing them (Fig. 1e, f). These tracheal cells showed heavy melanization (Fig. 1e, f, arrow), indicating that they were encapsulated (see Discussion). There was no evidence of invasion or further dispersal of the virus through the tracheal branches supplying the midgut (Fig. 1e, f). The simultaneous GFP signalling of the tracheal cells and the coincident melanization of it suggest that the limitation to spreading of the viral infection through the host is associated with the immune response of the latter.

**BV injection overcomes *S. littoralis* resistance to AcMNPV**

To further characterize the reaction of the host to the viral infection, we performed intrahaemocoelic injection of vHSGFP BVs into the proleg of fourth-instar *S. littoralis* larvae. About 90% of the virus-injected larvae had succumbed to the infection after 139 h (Fig. 2a). The dose–response curve of vHSGFP (Fig. 2b) was similar to that obtained by injection of AcMNPV (not shown). Approximately $10^2$ BVs caused mortality of 50% of the injected larval population.

To follow the spread of the vHSGFP baculovirus in the *S. littoralis* body, we monitored the appearance of GFP fluorescence upon BV injection. At 6–12 h post-infection, viral GFP-tagged foci without melanization were detected mainly at the tracheoblasts, tracheal branch joints servicing other tissues (Fig. 3a–c). After 24 h infection, the viral signal had spread through the tracheal epidermal cells (Fig. 3d). The infection had propagated successfully at 48 h post-infection through the tracheal branches (Fig. 3e, f), infecting other tissues [e.g. adjacent ganglia (Fig. 4a)], and culminated in a general infection of the larval fat-body tissue at 72 h post-infection (Fig. 4b, c). This later resulted in death of larvae, as evidenced by the high mortality rate obtained in parallel bioassays of injected BV vHSGFP (Fig. 2). The overall progression of the infection starting at 24 h is illustrated in the whole-body mounts of infected larvae (Fig. 5), showing viral foci spread through the trachea at 24 h post-infection (Fig. 5a, b) and expanding to the fat body and epidermis at 48 and 72 h post-infection (Fig. 5c–f). In contrast, mock-infected larvae survived and showed no GFP signalling (Fig. 5g, h). Unexpectedly, *S. littoralis* haemocytes appeared resistant to infection, with only 4–13% of them showing GFP signalling between 14 and 96 h post-infection (Fig. 6).

From these experiments, we concluded that the principal targets for initiation of haemocoelic viral infection were the tracheoblasts, with virus infection propagated through epidermal cells of the tracheal branches to adjacent tissues (e.g. fat-body cells, ganglia and body-wall cells); the haemocytes showed low propensity or were resistant to the infection.

**Immunosuppression of *S. littoralis* enhances AcMNPV infection**

The above data suggested involvement of the insect immune system in suppressing or attenuating the AcMNPV baculovirus infection. *S. littoralis* larvae were susceptible to initial infection at the midgut-cell level, but immune responses were activated by infection that subsequently led to melanization and viral clearance. Virus-infected *S. littoralis* haemocytes were less common than expected and may have a role in suppression or limiting virus expression and replication.

To further investigate the immune barriers to BV infection, two approaches were utilized. First, *S. littoralis* larvae were parasitized by the endoparasitic wasp *Chelonus inanitus*, which carries the PDV CiBV. This parasitization event delivers a PDV and associated venom proteins that produce a generalized suppression of host immune responses (Wyder et al., 2003). These parasitized larvae were subsequently injected with AcMNPV BV. Second, in a more refined approach, a single gene from the vankyrin family of genes of PDVs, *P*-vank-1, was delivered to *S. littoralis* larvae. This gene is thought to be involved in blocking the NF-κB...
signal-transduction cascade of the immune response (Kroemer & Webb, 2005).

To examine effects of parasitization, *S. littoralis* eggs were exposed to *Chelonus inanitus* and the parasitized larvae were allowed to develop to the fourth instar. After moultling to the fourth instar, larvae were injected with various doses of AcMNPV BV (Fig. 7). The speed of kill of equivalent doses of AcMNPV was faster in *Chelonus inanitus*-parasitized *S. littoralis* larvae than in immunocompetent larvae or mock-infected parasitized larvae (compare, for example, the speed of kill of 1000 and 100 p.f.u. AcMNPV in parasitized larvae with the speed of kill of 1000 p.f.u. AcMNPV in immunocompetent larvae; Fig. 7a). Moreover, we compared the proportion of haemocytes showing GFP signalling in immunosuppressed and untreated *S. littoralis* larvae infected with vHSGFP BV. It can be seen that AcMNPV-infected, immunosuppressed larvae exhibited a greater than twofold-higher percentage of infected haemocytes than did immunocompetent larvae (Fig. 7b). However, even in parasitized larvae, the majority of haemocytes did not show signs of baculovirus infection or fluorescence.

To investigate the effect of a single, putatively immunosuppressive CsIV gene, we injected recently moulted fourth-instar *S. littoralis* larvae with increasing doses of vAcPvank-1. This recombinant baculovirus expressed the P-vank-1 immunosuppressive protein and was compared with wild-type AcMNPV BV. The dose of recombinant vAcPvank-1 virus required to cause 50% mortality in the *S. littoralis* population was 10-fold lower than the dose required for wild-type AcMNPV (Fig. 8).

**DISCUSSION**

*S. littoralis* larvae are highly resistant to oral infections with AcMNPV polyhedra (Du *et al*., 1999; this study). Closer examination of the physiological basis for this resistance
shows that *S. littoralis* fourth-instar larvae infected orally with vHSGFP respond to the viral challenge by encapsulating and melanizing the virus-infected tracheal cells, limiting the infection (Fig. 1). At 72 h post-infection, the viral foci were cleared by larval moulting, resulting in abortion of the infection. However, it appears that encapsulation is only one of the barriers to AcMNPV replication in *S. littoralis*. Haemocoelic injection of BV results in successful infection of the tracheoblasts, followed by spread of the virus through the tracheal epidermis (Figs 3, 5). This infection pathway supports the view that the tracheolar cells are an important gateway through which lepidopteran baculoviruses establish systemic infections (Engelhard *et al.*, 1994; Haas-Stapleton *et al.*, 2003; Kirkpatrick *et al.*, 1994). However, barriers to infection are evident even after haemocoelic infection of virus. Notably, viral infections in haemocytes were limited to a small percentage of total cells (Fig. 6). In contrast, in permissive hosts, tracheoblasts and haemocytes are equally susceptible to infection (Engelhard *et al.*, 1994). The resistance of haemocytes to productive infection is a strong indication that other aspects of the immune system of *S. littoralis* limit AcMNPV infection. Previously, we have shown that AcMNPV induces apoptosis of *S. littoralis* SL2 cells and this is yet another type of protective host response to viral infection (Chejanovsky & Gershburg, 1995). Moreover, Zhang *et al.* (2002) found that the haemocytes of AcMNPV-infected *Spodoptera litura* larvae (related closely to *S. littoralis*) undergo apoptosis in response to AcMNPV infection. The extent of involvement of haemocytes from another spodopteran species, *S. frugiperda*, is more controversial, apparently due to technical differences in the manipulation of the viral inoculum that influence the ability of these cells to support AcMNPV replication (Clarke & Clem, 2002; Haas-Stapleton *et al.*, 2003). Our finding that general immunosuppression of the host, mediated by *Chelonus inanitus* inoculation of a PDV, increased the percentage of haemocytes showing GFP signalling (Fig. 7b) supports the concept that the haemocytes ‘react’ to some viruses and, in this instance, block the propagation of AcMNPV. Alternatively, it is also possible that, whilst the haemocytes are equally susceptible to infection in immunocompetent and immunosuppressed larvae, the titre of the baculovirus in the haemolymph of the latter host is higher, due to some other effect of immunosuppression on the insect, resulting in more haemocytes becoming infected.

Previous experiments have demonstrated that immunosuppression enhances AcMNPV infection of a non-permissive host (Engelhard *et al.*, 1994; Kirkpatrick *et al.*, 1994; Washburn *et al.*, 1996, 2000). In this study, we have identified a specific PDV gene, *P-vank-1*, that can improve AcMNPV infectivity when expressed from the baculovirus itself (Fig. 8). Some PDV vankyrins inhibit immune-system transduction signalling by mimicking IκB inhibition of the Dorsal/NF-κB transcription factors (Thoetkiattikul *et al.*, 2005). In CsIV, there is differential expression of members of the vankyrin gene family, with *P-vank-1* expressed preferentially in the fat-body tissues of *Heliothis virescens* larvae parasitized with *Campoletis sonorensis* and a second vankyrin gene, *P-vank-3*, expressed preferentially in the haemocytes (Kroemer & Webb, 2005). Thus, it will be interesting to assess the impact of other CsIV vankyrins on progression of AcMNPV infections in *S. littoralis* fat body and haemocytes.

This line of investigation will help to elucidate the mechanisms through which the immunosuppressive PDVs, such as CsIV and CiBV, promote replication of other well-characterized pathogenic viruses, such as AcMNPV, in...
lepidopteran hosts that are semi- or non-permissive to virus replication.

In conclusion, this report demonstrates that *S. littoralis* larvae mount immune responses that limit AcMNPV at the gut level and at the haemocyte level. The data suggest that at least two separable components of the immune system, the cellular and the humoral aspects, are involved in this immune response and that haemocytes may play a central role in limiting virus infection in this system.

**ACKNOWLEDGEMENTS**

We thank Rollie J. Clem from Kansas State University, USA, who kindly provided us with vHSGFP, Beatrice Lanzrein, University of Berne, Switzerland, who kindly provided the *Chelonus inanitus* wasps and David Nestel from The Volcani Center, Israel, for his assistance in statistical analysis of the data. We acknowledge support for this research by the BARD, under grant no. IS-3783-05C to N.C and B.A.W. Contribution from the Agricultural Research

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**Fig. 5.** BV infection of *S. littoralis* larvae with vHSGFP, binocular picture. Left panels, whole-body mounts of infected larvae; right panels, GFP fluorescence. (a, b) 24 h post-infection, magnification ×80; (c, d) 48 h post-infection, magnification ×40; (e, f) 72 h post-infection; (g, h) mock-infected larvae, magnification ×8.

**Fig. 6.** Percentage of haemocytes showing GFP expression in fourth-instars *S. littoralis* larvae injected with 2 × 10⁴ vHSGFP BV. Cohorts of 10 caterpillars were sacrificed at each time point for haemolymph collection. The percentage of GFP-positive haemocytes in each sample was determined and plotted as mean ± SD.
REFERENCES


Fig. 7. (a) Time response of Chelonus inanitus-parasitized (wasp) S. littoralis larvae to AcMNPV infection (Ac). Larval mortality of 30 cohorts per sample challenged with the indicated BV doses (10–100 m.o.i.). S. littoralis eggs were exposed to Chelonus inanitus and transferred to individual cups. AcMNPV BV injections were performed with recently ec lysed fourth-instar larvae (the experiment was repeated in triplicate). (b) GFP-positive haemocytes in Chelonus inanitus-parasitized and non-parasitized S. littoralis at various times after infection with vHSGFP (Wasp+vHSGFP and vHSGFP, respectively). Wasp+vHSGFP treatment differed significantly from vHSGFP treatment (F,1,43 = 20.03, P<0.01, general linear models, means separated by LSD; Statgraphics, 2000).

Fig. 8. Response of S. littoralis larvae to vAcPvank-1 and AcMNPV-infection. Larval mortality of 30 cohorts per sample challenged with the indicated BV doses (the experiment was repeated in triplicate).


