Apoptosis is induced in the haemolymph and fat body of *Spodoptera exigua* larvae upon oral inoculation with *Spodoptera litura* nucleopolyhedrovirus

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*Spodoptera exigua* multinucleopolyhedrovirus (SeMNPV) and *Spodoptera litura* nucleopolyhedrovirus (SpltNPV) are genetically similar, but the larvae of *S. exigua* are not susceptible to SpltNPV. The aim of this study was to identify whether any process was inhibiting SpltNPV infection at some point. *S. exigua* larvae infected with a high concentration of wild-type SpltNPV by oral inoculation produced a fatal infection in second- or third-instar *S. exigua*, but the dead larvae did not undergo liquefaction; in contrast, fourth-instar infected larvae remained healthy. RT-PCR analysis of total RNA from infected second-instar larvae targeting immediate-early (*ie-0*), early (*dnapol*), late (*chit*) and very late (*polh*) genes suggested that SpltNPV initiated infection in the non-susceptible hosts. Total DNA extracted from the haemocytes of infected larvae showed DNA ladders characteristic of apoptosis. Sections of tissue from infected third-instar larvae of *S. exigua* at 96 h post-inoculation, stained with haematoxylin and eosin, revealed a highly disrupted morphology in the fat body. Apoptosis in fat body tissue was detected using terminal deoxynucleotidyltransferase-mediated fluorescein–dUTP nick end labelling (TUNEL) assays. *In situ* hybridization revealed the presence of viral DNA within the TUNEL-positive area, indicating viral infection in this tissue. These results suggest that apoptosis limits viral propagation by reducing the number of SpltNPV-infected haemocytes and fat body cells and inhibits disseminated viral infection.

INTRODUCTION

The family *Baculoviridae* comprises a diverse group of arthropod-specific viruses and contains two genera, *Nucleopolyhedrovirus* (NPV) and *Granulovirus*, based on their occlusion body morphology (Theilmann *et al.*, 2005). Baculoviruses are characterized by a circular double-stranded DNA genome packaged within a rod-shaped capsid and enclosed by a lipid envelope. During a single infection cycle, baculovirus virions are present in two forms: occlusion-derived virus (ODV) and budded virus (BV). ODVs are embedded in large protein crystals known as polyhedra or occlusion bodies (OBs). Once polyhedra are ingested by the larvae and dissolved in the alkaline juices in the midgut, ODVs are released and pass through the peritrophic membrane. The virion envelope fuses with the membrane of columnar midgut epithelial cells, allowing the nucleocapsids to enter the microvilli to initiate the primary infection (Federici, 1997). In lepidopterans, the NPVs establish a transient infection in the midgut, without producing OBs; progeny nucleocapsids bud from the cell at the basal site and become BVs. The haemocoel (Granados & Lawler, 1981; Keddie *et al.*, 1989; Federici, 1997) and/or the tracheal system (Engelhard *et al.*, 1994; Rivkin *et al.*, 2006) then circulate the BVs throughout the body, facilitating rapid transmission and infection in other tissues. NPVs can attack most tissues in their lepidopteran hosts, replicating particularly well in the fat body, tracheal matrix and epidermis in systemic infections. At later stages of infection, the dead caterpillar is liquefied to release billions of polyhedra, which transmit the virus horizontally to susceptible hosts (Federici, 1997).

Baculoviruses have been reported worldwide from over 700 host species and have been used as bioinsecticides against many insect pests, mainly *Lepidoptera* (Moscardi, 1999). Baculoviruses have limited host ranges, usually being
restricted to one host species or genus (Miller & Lu, 1997). Understanding the nature of such host-range restrictions will allow more accurate predictions regarding the potential effects of modifying the viral genomes and the impact of such modifications on the ability of the viruses to infect beneficial or non-target organisms (Miller & Lu, 1997). Studies on the nature of these blocks have been pursued at a variety of levels and apoptosis is thought to be one of the factors affecting the host range of baculoviruses.

Apoptosis is an active process of cellular self-destruction, characterized by a number of distinct morphological and biochemical features including cellular shrinkage, plasma membrane blebbing, chromatin condensation and internucleosomal cleavage (White, 1996). It can serve an essential function for multicellular organisms by eliminating cells that might prove harmful to the organism as a whole, e.g. targeting virus-infected cells for apoptotic cell death to protect animals from virus attack (O’Brien, 1998). Baculoviruses induce apoptosis in a host-specific manner. Wild-type Autographa californica multinucleopolyhedrovirus (AcMNPV) replicates normally in Sf-21 cells derived from the fall armyworm, Spodoptera frugiperda; however, it induces apoptosis in SI-2 cells derived from the Egyptian cotton worm, Spodoptera littoralis (Chejanovsky & Gershburg, 1995), in SI-ZSU-1 cells derived from the cotton leaf worm, Spodoptera litura (Dai et al., 1998), and in Cf-203 cells derived from the eastern spruce budworm, Choristoneura fumiferana (Palli et al., 1996). Baculoviruses have evolved two different anti-apoptotic genes, p35 and iap, to prevent host apoptosis (Clem, 2001). An AcMNPV p35 deletion mutant can induce apoptosis in Sf-21 cells; however, such a mutant can allow productive infection in Tn-368 cells from the cabbage looper, Trichoplusia ni (Clem et al., 1991). The production of viral progeny is significantly lower in Sf-21 cells infected with p35 mutants than in cells infected with wild-type AcMNPV (Clem & Miller, 1993). These results show that the anti-apoptotic genes are host-range determinants.

Although many investigations of baculovirus infections within susceptible insect hosts have been described, our understanding of the mechanisms of failure of baculoviral infection within non- or semi-permissive insect hosts lags behind. Apoptosis is hypothesized to play an important role in insect immunity to baculovirus infection (Clarke & Clem, 2003a). Recently, in vivo baculovirus induction of apoptosis has been reported in two baculovirus–host systems: (i) wild-type AcMNPV-induced apoptosis in S. litura larvae (Zhang et al., 2002) and (ii) p35 mutant AcMNPV-induced apoptosis in S. frugiperda larvae (Clarke & Clem, 2003b). However, in both studies, the experimental larvae were infected by intraheamocoeelic injection, in contrast to the typical natural oral route of infection. To the best of our knowledge, there have been no reports of in vivo apoptosis induced by baculovirus oral infection to date.

In the present study, we investigated the progression of S. litura nucleopolyhedrovirus (SpltNPV) infection in the non-permissive host S. exigua. The complete genome sequence of SpltNPV has been reported (Pang et al., 2001). Our preliminary data indicated that S. exigua larvae were completely resistant to oral infection following ingestion of SpltNPV polyhedra. Baculovirus-induced apoptosis of larvae has not been studied extensively. The aim of this study was to detect SpltNPV-induced apoptosis in the tissues of S. exigua larvae. Here we report that in vivo apoptosis of S. exigua larvae was triggered by oral inoculation with wild-type SpltNPV, demonstrating for the first time that apoptosis can be induced by baculovirus oral infection and confirming that apoptosis is a limiting factor for virus propagation in non-susceptible host(s).

**METHODS**

**Insects, cell lines and viruses.** S. exigua and S. litura larvae were reared on an artificial diet (Li et al., 2002) and maintained at 27 °C under a constant photoperiod (14 h light and 10 h dark) and humidity (60%). The S. litura cell line TUAT-SpL221 (SpL-221) (Yanase et al., 1998) was donated by Dr Su Zhihui (JT Biotechnology Research Hall, Osaka, Japan) and cultured at 27 °C in Grace’s insect medium (Invitrogen) supplemented with 10% fetal bovine serum, glutamine (2 mmol l⁻¹), penicillin (100 U ml⁻¹) and streptomycin (100 U ml⁻¹). The SpltNPV G2 strain was originally isolated from infected larvae of S. litura in Guangzhou, China, in 1976 (Pang et al., 2001). OBs were propagated in fourth-instar S. litura larvae and purified as described previously (Simon et al., 2004).

**Inoculation of larvae.** To determine the infectivity of SpltNPV, S. exigua larvae were inoculated orally with SpltNPV at different developmental stages. Second- or third-instar larvae were inoculated by the droplet feeding method as described previously (Hughes & Wood, 1981). Briefly, newly moulted larvae were starved for 6 h and then inoculated with a solution containing 1% sucrose (w/v), 0.01% blue food colouring (FD&C Blue No. 1) and 10⁶ OBs ml⁻¹. Each second- or third-instar larva ingested 0.25 ± 0.03 μl (about 2.5 × 10⁶ OBs) or 0.57 ± 0.03 μl (about 5.7 × 10⁵ OBs), respectively. Each fourth-instar newly moulted larva was inoculated orally with 2 μl OBs (about 2 × 10⁶ OBs) using a syringe with a blunt-tipped needle. Inoculated larvae were maintained individually in 1 oz plastic cups and provided with an artificial diet. Experiments were carried out in triplicate with 35 larvae per treatment. Larvae were observed daily until death or pupation. Negative-control larvae were treated identically with solutions not containing SpltNPV; SpltNPV-infected S. litura larvae were used as a positive infection control.

**RNA extraction from larvae.** Total RNA was extracted using the E.Z.N.A. Total RNA kit II (Omega Bio-Tek) following the manufacturer’s protocol. Whole inoculated larvae used for detection of SpltNPV transcripts were disrupted and homogenized directly in RNA-Solv reagent (Omega Bio-Tek) (30 mg tissue ml⁻¹). The tube containing homogenate was incubated at room temperature for 5 min; samples were mixed with 200 μl chloroform, vortexed for 20 s, incubated on ice for 15 min and centrifuged at 12 000 g at 4 °C for 15 min to separate the aqueous and organic phase. The upper aqueous phase (~500 μl) was transferred into a new 1.5 ml centrifuge tube, mixed with an equal volume of 70% ethanol and mixed thoroughly by vortexing. The sample was applied to a HiBind RNA spin column (Omega Bio-Tek) and washed with RNA Wash Buffers I and II. The column was centrifuged for 1 min at full speed. The RNA was eluted with 30 μl DEPC-treated water, quantified by measuring the absorbance at 260 nm in a spectrophotometer and stored at
Detection of viral transcripts. RT-PCR was performed to detect viral gene transcripts in the insect larvae. Total RNA was digested with RQ1 RNase-free DNase (Promega). Equivalent amounts of RNA (0.25 μg) were used in each reaction. A PCR was performed to verify the absence of contaminant DNA in these samples. RT-PCR was performed using a Takara RNA-PCR kit (AMV) version 3.0 according to the manufacture’s protocol. First, cDNA was synthesized using the avian myeloblastosis virus reverse transcriptase and oligo(dT) primers. Subsequently, cDNA fragments were amplified with Tag Polymerase DNA polymerase and the forward and reverse primer mixture for the target gene (see Supplementary Table S1, available in JGV Online). PCR products were analysed by 1% agarose gel electrophoresis. DL2000 marker (TakaRa) containing 0.2–2 kb fragments was used for size determination. DNA fragments were stained with ethidium bromide, photographed and examined using the Electrophoreses Documentation and Analysis System 120 (Kodak).

Insect histopathology. Third-instar larvae of S. exigua infected with SpltNPV at 96 h post-inoculation (p.i.) were fixed in 4% paraformaldehyde for 24 h. The larvae were dehydrated in graded ethanol to xylene and embedded in paraffin. Sections were cut cross-sectionally at a thickness of 6 μm and stained using either haematoxylin/eosin (H&E) for larval tissue (Ausubel et al., 1995) or Hamm’s method for polyhedra (Hamm, 1966). The stained sections were observed and photographed using an optical microscope (ECLIPSE TE2000 U; Nikon).

Apoptosis assays

DNA fragmentation. Apoptotic DNA fragments were isolated according to the method described by Herrmann et al. (1994). The larval haemocytes of S. exigua were collected at 96 h p.i., washed with PBS (pH 7.2) containing 0.1% dithiothreitol and centrifuged for 1 min at 1000 g. The pellets were then incubated in lysis buffer [1% NP-40 in 20 mM EDTA, 50 mM Tris/HCl (pH 7.5)] for 5 min. After centrifugation for 5 min at 1600 g, the supernatants were collected. SDS and RNase A were added to final concentrations of 1% and 5 μg ml⁻¹, respectively. After 2 h treatment at 56 °C, the supernatants were digested with proteinase K (final concentration 2.5 μg ml⁻¹) at 37 °C for 2 h. The DNA was precipitated with ethanol, dissolved in TE and separated by electrophoresis in 1.5% agarose gels.

In situ cell death detection. An assay was performed using an In situ Cell Death Detection kit (Roche) according to the manufacturer’s protocol. The paraffin-embedded sections were dewaxed in xylene and rehydrated in serially graded ethanol, then treated with proteinase K (5 μg ml⁻¹) in PBS for 30 min at 37 °C. After two washes with PBS, the sections were treated with 50 μl terminal dUTP nick end labelling (TUNEL) reaction mixture containing terminal deoxynucleotidyltransferase and fluorescein-dUTP for 1 h at 37 °C in the dark. After rinsing three times with PBS, sections were observed by fluorescence microscopy (ECLIPSE TE2000 U; Nikon).

In situ DNA fragmentation. Sections were stained with 4',6-diamidino-2' -phenylindole dihydrochloride (DAPI; Roche Applied Science) at a final concentration of 1 μg ml⁻¹ for 15 min at 37 °C in the dark. DAPI associates with the minor groove of dsDNA, a sign of intact nuclei, and fluoresces blue upon UV excitation. Sections were rinsed twice with methanol and observed by fluorescence microscopy (360/340 nm excitation, 460/450 nm emission).

In situ hybridization. Virus DNA in paraffin sections was detected by in situ hybridization using a digoxigenin-labelled probe complementary to the virus genomic DNA. A PCR-amplified oligonucleotide of the SpltNPV polyhedrin gene (Supplementary Table S1) was labelled by terminal transerase using a DIG DNA Labelling and Detection kit (Roche) according to the manufacturer’s protocol. To study the presence of SpltNPV in the fat body, third-instar larvae infected with SpltNPV at 96 h p.i. were fixed in 4% paraformaldehyde in PBS at 4 °C overnight. Consecutive 4 μm sections were mounted onto 3-aminopropyltriethoxysilane-coated slides and dried at 37 °C overnight. The tissues were dewaxed in fresh xylene (twice for 10 min each) and rehydrated through a graded ethanol series. The sections were digested with proteinase K (3 μg ml⁻¹; Roche) in TE buffer at 37 °C for 20 min. Following post-fixation in 4% paraformaldehyde for 5 min, acetylation was performed to prevent non-specific binding of the probe to sections.

The sections were pre-hybridized in hybridization buffer (1× Denhardt’s solution, 5% dextran sulphate, 200 μg sonicated salmon sperm DNA ml⁻¹, 4× SSC, 50% formamide) for 1 h at 42 °C, followed by the addition of digoxigenin-labelled probe at a concentration of 1 μg ml⁻¹. The sections were placed on a heating plate at 95 °C for 6 min and cooled on ice for 1 min, then hybridized for 16 h at 42 °C. After hybridization, the sections were washed twice in 2× SSC for 5 min and once in 0.1× SSC for 10 min. A positive hybridization signal was detected using alkaline phosphatase-conjugated anti-digoxigenin antibody at a 1:500 dilution for 1 h at room temperature. At the end of the signal development, the sections were washed in PBS and mounted. Images were captured on a Nikon microscope using a Nikon digital camera DXM1200 F and ACT-1 (Nikon).

Haemolymph virus assay. To determine whether SpltNPV was able to produce a secondary infection, haemolymph BV titres from virus-infected larvae were assayed. Larvae were chilled for 30 min at 4 °C before haemolymph extraction. Haemolymph was collected every 24 h by cutting an anterior proleg and allowing the larvae to bleed into ice-cold Grace’s medium supplemented with 8 mM dithiothreitol. Samples were centrifuged at 800 g for 10 min at 4 °C; supernatant samples were sterilized through a 0.45 μm Millipore filter and stored at −80 °C for determination of virus levels. BV titres were assayed by a TCID₅₀ end-point dilution assay on SpLi-221 cells (O’Reilly et al., 1992). Three replicates were scored for each sample.

RESULTS

Gross pathology of S. exigua infected with SpltNPV

First, the infectivity of SpltNPV in second-instar S. exigua larvae was determined following oral inoculation at different concentrations. When the larvae were inoculated with 10⁶ OBs ml⁻¹, they did not exhibit any characteristics of baculovirus disease and could moult and survive to pupae normally. When challenged with an inoculum concentration of 10⁷ OBs ml⁻¹, the percentage of dead larvae was 29.1% at 10 days p.i.; 70.9% of infected larvae displayed mortal infection when challenged with 10⁸ OBs ml⁻¹ at 6 days p.i. When a high concentration (10⁹ OBs ml⁻¹) was administrated to the larvae, the body mass (Fig. 1a) and body length (Fig. 1b) of the infected larvae decreased dramatically over the course of infection compared with the developmentally matched uninfected larvae. This difference could be seen clearly at 72 h p.i. The mean length and mass (n=35, triplicate experiments) of uninfected larvae were significantly greater than those of...
infected larvae. By 96 h p.i., the uninfected larvae were 46% longer and 92% heavier than the infected larvae (Fig. 1). Infected larvae started to die at 96 h p.i. (10.5%) and 61.9% of infected larvae were dead by 120 h p.i. By 144 h p.i., the percentage of dead larvae reached 84.8%. None of the dead larvae underwent liquefaction, a typical symptom associated with fatal baculovirus infection. The results showed significant differences in sensitivity when second-instar S. exigua larvae were inoculated orally with SpltNPV at different concentrations.

Next, we took advantage of the high concentration of SpltNPV (10^9 OBs ml⁻¹) to challenge later-instar S. exigua larvae. Generally, SpltNPV produced atypical symptoms in third-instar S. exigua larvae; in contrast, fourth-instar larvae did not become infected. For the third-instar larvae, the growth of the inoculated larvae was also very slow (data not shown); the larvae could survive up to 10 days and the dead larvae did not liquefy. Fourth-instar infected larvae did not show any pathological symptoms and could moult to pupae normally. These results indicated that early-instar larvae were more susceptible to the virus than the later-instar larvae.

Detection of SpltNPV transcripts in S. exigua larvae

To determine whether SpltNPV could enter midgut epithelial cells and established an infection in the non-permissive S. exigua host, we designed specific primers for the immediate-early ie-0 gene, the early DNA polymerase (dnapol) gene, the late chitinase (chit) gene and the very late polyhedrin (polh) gene of SpltNPV (Supplementary Table S1) to detect the presence of viral transcripts. RT-PCR was performed on total RNA isolated from whole, infected, second-instar S. exigua larvae at 6, 12, 24 and 48 h p.i. Larvae treated identically with solutions not containing SpltNPV OBs were used as a negative control, and SpltNPV-infected S. litura larvae were used as a positive control.

RT-PCR analysis of total RNA isolated from inoculated larvae showed that SpltNPV initiated an infection in S. exigua larvae. A transcript of the SpltNPV ie-0 gene was not detected at 6 h p.i. in S. exigua larvae. Compared with SpltNPV-infected S. litura larvae, transcription of the ie-0 gene was delayed and the quantity of transcript was lower in S. exigua (Fig. 2a). To determine whether the cascade of gene expression was blocked at any point in S. exigua larvae, transcription of an early (dnapol), a late (chit) and a very late (polh) gene was studied. RT-PCR results indicated the presence of specific transcripts of these three genes in the non-susceptible host (Fig. 2); however, the transcriptional activity of dnapol, chit and polh was lower in S. exigua than in S. litura.

Apoptosis in the tissues of SpltNPV-infected S. exigua larvae

The above results indicated that SpltNPV could initiate an infection in second-instar S. exigua upon oral inoculation. As SpltNPV could also cause atypical symptoms in third-instar S. exigua larvae, this suggested that the virus could establish an infection in this developmental larval stage. As fourth-instar larvae resisted SpltNPV infection, we used third-instar larvae to investigate whether apoptosis could play a role in inhibiting SpltNPV infection.

Apoptosis in haemocytes. To investigate whether apoptosis was induced in the haemolymph of S. exigua larvae upon oral inoculation with baculovirus, haemolymph was extracted from infected S. exigua larvae at 96 h p.i. and observed under an optical microscope. Significant differences were detected in the haemocytes from uninfected and infected S. exigua larvae. The haemocytes of infected S. exigua larvae showed blebbing (Fig. 3b), whilst those of uninfected S. exigua larvae exhibited their characteristic round shape (Fig. 3a).

Further experiments were performed to detect the presence of DNA ladders. Haemolymph was collected from a cohort of 20 larvae (about 100 μl) at 96 h p.i. and the haemocytes were separated by centrifugation. Total DNA was extracted.
from the haemocytes. A DNA ladder was observed after electrophoresis of the infected *S. exigua* DNA sample. However, no DNA ladder was detected from mock-infected *S. exigua* larvae at 96 h p.i. (Fig. 3c).

**Apoptosis in the fat body.** Tissue sections were obtained from paraformaldehyde-fixed, paraffin-embedded SpltNPV-infected third-instar larvae at 96 h p.i. and stained with H&E for histopathological analysis. Samples from uninfected control larvae exhibited normal fat body morphology (Fig. 4a). Tissues from SpltNPV-infected *S. litura* larvae displayed characteristics typical of baculovirus pathology, with the tissues being distinctly swollen and the nuclei of fat body cells having increased diameter (Fig. 4b).

In contrast, the fat body from SpltNPV-infected *S. exigua* larvae showed a strongly disrupted morphology consisting of the presence of cavities and pores in the normally contiguous fat body and condensed regions of darkly staining tissue (Fig. 4c). We assumed these cavities and pores to be caused by apoptosis induced by viral infection, as the apoptotic cells shrank and became round, detaching themselves from adjacent cells, and the resulting free apoptotic bodies were phagocytosed by macrophages or internalized by adjacent healthy cells.

When Hamm’s staining was applied to sections of larvae infected with a nucleopolyhedrovirus, the polyhedra stained bright red. As expected, in SpltNPV-infected *S. litura* larvae tissue, fat body cells were detected with polyhedra stained red in the swollen nuclei (Fig. 4e, arrows). The fat body of uninfected *S. exigua* larvae remained normal, with the fat body cells staining green and no red cells being found (Fig. 4d). In contrast, faint red cells were distributed randomly in SpltNPV-infected *S. exigua* larvae, implying that a few OBs or polyhedra, although not as many as in SpltNPV-infected *S. litura*, might be formed or synthesized in the non-susceptible host (Fig. 4f, arrows).

To determine whether apoptosis occurred in the tissues of virus-infected *S. exigua* larvae, TUNEL staining was performed using an *In situ* Cell Death Detection kit. In SpltNPV-infected *S. exigua* larvae at 96 h p.i., extensive numbers of TUNEL-positive cells were observed in fat body tissue, suggesting internucleosomal cleavage of DNA.
In sections from SpltNPV-infected S. exigua larvae at 48, 72 and 120 h p.i., TUNEL-positive staining was also observed in the fat body (data not shown). The apoptosis-positive cells were alone or in small clusters located in the fat body (Fig. 5l). Counterstaining of sections with DAPI showed that the majority of TUNEL-positive nuclei were condensed and fragmented, confirming apoptotic cell death (Fig. 5i). H&E, DAPI and TUNEL staining of SpltNPV-infected larvae demonstrated that extensive apoptosis was occurring in fat body tissue.

In sections from uninfected S. exigua larvae at 96 h p.i., TUNEL-positive cells were occasionally observed, indicating a low level of background apoptosis occurring in the larvae tissue (Fig. 5j). Sections from SpltNPV-infected S. litura larvae at 96 h p.i contained small numbers of scattered TUNEL-positive cells, but the intensity was significantly weaker than that observed in infected S. exigua larvae (Fig. 5k).

To confirm SpltNPV infection in the fat body of infected S. exigua larvae, in situ hybridization experiments were performed using digoxigenin-labelled probes. No labelling was found in tissues from uninfected S. exigua larvae (Fig. 5d). A strong positive signal was observed in tissue from SpltNPV-infected S. litura larvae (Fig. 5e). A weak positive hybridization signal was detected in the fat body from SpltNPV-infected S. exigua larvae at 96 h p.i. (Fig. 5f), indicating that SpltNPV had established an infection in the fat body. Due to apoptosis, DNA fragmentation occurred in fat body cells from SpltNPV-infected S. exigua larvae. As a result of apoptosis, larger numbers of DNA fragments were degraded and thus only small numbers of viral DNA fragments were found in fat body tissue and the hybridization signal was weaker than that of SpltNPV-infected S. litura.

**Haemolymph virus level**

Although fourth-instar S. exigua larvae could pupate normally after challenge with high concentrations of SpltNPV, we were interested in knowing whether SpltNPV could establish an infection in these older instars. Fourth-instar S. exigua larvae were infected orally with $10^9$ OBs ml$^{-1}$ and BV titres from haemolymph at different time points were determined by a TCID$_{50}$ end-point dilution assay on SpLi-221 cells, which are susceptible to SpltNPV infection. SpltNPV-infected S. litura larvae were used as a positive infection control. The infected S. exigua larvae exhibited a rapid build-up of virus in the haemolymph by 48 h; the level of virus remained the same up to 72 h, but decreased by 96 h (Fig. 6). By contrast, the infected S. litura larvae also exhibited a rapid build-up of virus in the haemolymph by 48 h, and the virus level in the haemolymph remained high throughout the course of infection. The infected S. exigua larvae had levels of virus in the haemolymph below the threshold of detection in the assay used at 24 h p.i. No OBs of SpltNPV could be visualized in the haemolymph of the inoculated fourth-instar larvae by optical microscopy.

**DISCUSSION**

Although a few studies have indicated that apoptosis could be a response to baculovirus infection to reduce viral...
infectivity in vivo (Zhang et al., 2002; Clarke & Clem, 2003a, b), this report, for the first time, provides evidence for the presence of apoptosis in insects upon oral inoculation with wild-type baculovirus (the natural route of baculovirus infection), demonstrating the role of apoptosis as an antiviral mechanism in the insect immune system. SpltNPV and SeMNPV present high similarity at the genome level (Ijkel et al., 1999; Pang et al., 2001; Herniou et al., 2003). The SpltNPV genome contains 141 putative open reading frames (ORFs) and the SeMNPV genome contains 139 ORFs, with 105 ORFs in common. S. exigua and S. litura are both extremely serious agricultural lepidopteran pests and belong to the same genera. Although both pathogens and hosts in the present study are closely related, preliminary results showed that the larvae of S. exigua were non-susceptible to SpltNPV. To establish a productive infection, NPVs must initiate a midgut infection within midgut epithelia. Progeny virions must then overcome a midgut basal lamina to disseminate into the tracheal system and/or the haemocoel and eventually infect and multiply within target organs, such as the fat body, epidermis and tracheal matrix. Failure to undergo productive infection indicates that restrictions to baculovirus replication are probably encountered, such as:

**Fig. 5.** Tissue sections of S. exigua larvae abdomens. S. exigua (Se) larvae were left uninfected (a, d, g, j) or infected with SpltNPV (c, f, i, l), or S. litura (Splt) larvae were infected with SpltNPV (b, e, h, k). All larvae were sacrificed at 96 h p.i. Sections were hybridized with digoxigenin-labelled oligonucleotide probes (d–f), stained with DAPI (g–i) or a TUNEL assay was carried out with fluorescein-labelled dUTP (j–l). Photographs were also taken under normal light (a–c). fb, Fat body. Bars, 100 μm.
In order to determine whether SpltNPV could establish infection in the non-susceptible host S. exigua by oral inoculation, we performed RT-PCR to examine the presence of transcripts at various times of infection, including immediate-early, early, late and very late phases. The results of these experiments showed that all gene transcripts could be detected, indicating that SpltNPV could replicate successfully in SpltNPV-infected S. exigua larvae (Fig. 2).

In addition, the BV titre assay showed a low level of virus in the haemolymph, confirming that progeny virus could be produced and could traverse the midgut basal lamina during infection (Fig. 6). These results suggest that the host response against viral entry into the midgut and passage through the basal lamina may be less efficient in aborting infection in S. exigua larval early instars compared with late instars. Recently, Simon et al. (2004) arrived at a similar conclusion: that SeMNPV was able to replicate in the two non-susceptible hosts S. frugiperda and S. littoralis and that all genes required for viral replication were present in the SeMNPV-infected larvae.

Histological studies were used to determine the changes in the infected S. exigua larval tissue. Fat body tissue of infected larvae was destroyed, which was evident from the appearance of many cavities and pores (Fig. 4c). In situ hybridization experiments demonstrated that SpltNPV could invade fat body cells (Fig. 5f). Sections stained using Hamm’s method showed that a few OBs seemed to be formed in fat body tissue (Fig. 4f). A TUNEL assay and counterstaining of sections with DAPI demonstrated that apoptotic cell death occurred in the damaged regions (Fig. 5). The cavities and pores may indicate that the infected cells underwent apoptosis and the resulting free apoptotic bodies were phagocytosed by macrophages or internalized by adjacent healthy cells. Therefore, we suggest that apoptosis may be an important barrier in limiting SpltNPV infection in S. exigua early-instar larvae.

Little is known about the mechanisms responsible for antiviral immunity in insects. However, several types of response of insects to baculovirus infection have been described over the past two decades, including: (i) sloughing or moulting to rid the host of infected midgut epithelial cells before the virus can penetrate the basal lamina (Keddie et al., 1989; Hoover et al., 2000); (ii) encapsulation and melanization of the virus-infected cells and tissues (Washburn et al., 1996, 2000; Rivkin et al., 2006); (iii) the haemocytes being highly resistant to viral infection (Trudeau et al., 2001; Clarke & Clem, 2002; Rivkin et al., 2006); and (iv) apoptosis occurring in infected cells or tissues (Zhang et al., 2002; Clarke & Clem, 2003a). In the present study, we showed that apoptosis was induced in the fat body and haemocoel, which may be responsible for the reduction in virus infectivity and the limitation of viral propagation. However, we cannot exclude the possibility that other aspects of the immune system of S. exigua limit SpltNPV infection. We found that the percentage of infected haemocytes was less than 1 % when fourth-instar S. exigua larvae haemocoel was injected with SpltNPV BV, indicating S. exigua haemocytes are highly resistant to SpltNPV; in contrast, in the susceptible host S. littura, almost all haemocytes were infected (data not shown). We also found that the viral infectivity varied from instar to instar. Older larvae were more resistant to virus infection, indicating that they may have the ability to mount new antiviral defences. Thus, it appears that apoptosis is only one of the barriers to SpltNPV replication in S. exigua.

(i) a mechanism at the level of the midgut preventing primary infection within epithelia, (ii) some mechanism preventing BVs from passing through the basal lamina, or (iii) inefficient secondary infection.

S. exigua larvae remained healthy when infected with a lower dose of SpltNPV OBs. However, when we fed second-instar larvae a high concentration of $10^9$ OBs ml$^{-1}$ (such as might be encountered during epizootics), infected larvae stopped growing and 84.8 % of infected larvae eventually died. The body mass (Fig. 1a) and body length (Fig. 1b) of the infected larvae decreased dramatically and death appeared to be due to starvation. The cadavers did not liquefy, whereas liquefaction is a hallmark of polyhedrosis diseases. These observations are consistent with previous reports in which non-protective larvae were infected experimentally by injection of BV into the haemocoel or by feeding of OBs (Clem & Miller, 1993; Washburn et al., 1996, 2000; Trudeau et al., 2001; Clarke & Clem, 2002, 2003b; Zhang et al., 2002; Rivkin et al., 2006).

High concentrations of virus also caused fatal infection in third-instar S. exigua larvae. In contrast, fourth-instar larvae did not become infected, even if each larva was challenged with a concentration of $10^9$ OBs ml$^{-1}$. Thus, the infectivity of SpltNPV in S. exigua larvae is variable and depends on the dosage of inoculum and the developmental stage of the larvae.
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REFERENCES


