The response of newly established cell lines of *Spodoptera littoralis* to group I and group II baculoviruses

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Abstract *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) and *Spodoptera littoralis* multiple nucleopolyhedrovirus (SpliMNPV) belong to group I and group II nucleopolyhedroviruses, respectively and can replicate in a wide range of insect species. In this study, the ability of newly established *S. littoralis* cell lines to support replication of AcMNPV and SpliMNPV was examined. The microscopic observations showed that the *S. littoralis* cells infected with AcMNPV exhibited morphological changes such as cells breaking into small bodies and forming apoptosis-like bodies post-infection. Nuclear DNA fragmentation was observed in all AcMNPV-infected cell lines through DNA gel electrophoresis analysis. Therefore, the virus replication was unsuccessful in most of cells, which were able to abort the virus replication. On the other hand, cells that were infected with SpliMNPV did not show similar

morphological changes and no small bodies were formed. In addition, SpliMNPV succeeded to infect the cells, replicate, and form viral occlusion bodies inside the infected cells. In suspension culture, *S. littoralis* cells, which were infected with AcMNPV, accumulated as composed balls in shaker flasks after infection overnight, with cell density decreasing dramatically. In contrast, there was no cell clumping seen in the infected cells with SpliMNPV and the uninfected cells. In conclusion, the newly established embryonic *S. littoralis* cells were highly susceptible to SpliMNPV, whereas the cells were non-permissive to AcMNPV, yet they still underwent programmed cell death.

Keywords *Spodoptera littoralis* · AcMNPV · SpliMNPV · Apoptosis

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Introduction

Baculoviruses are a family of rod-shaped enveloped viruses, 40–50 nm in diameter and 200–400 nm in length, which are specific insect pathogens (Palomares et al. 2006; Clem and Passarelli 2013). Baculoviruses contain a double stranded, circular DNA genome, ranging from 80 to 200 Kbp (Jehle et al. 2006); they have been widely used to develop gene expression systems for recombinant protein production as well as in gene therapy. *Autographa californica* multiple

nucleopolyhedrovirus (AcMNPV) is one of the most important baculoviruses, as it is used in recombinant protein production (Patterson et al. 1995; Kost et al. 2005; Airenne et al. 2013). In 1983, AcMNPV was first used as an expression system to produce a recombinant protein in insect cells (Smith et al. 1983). AcMNPV has a wide insect host range, which can infect more than 73 species (Adams and McClintock 1991). Baculovirus genes have the ability to control insect cells and preclude them undergoing apoptosis. This process is controlled by anti-apoptotic genes, such as p35 and IAPs, which play an essential role in blocking apoptosis (Clem et al. 1991; Seshagiri and Miller 1997; Clem 2001).

Apoptosis is a programmed cell death in which cells undergo dramatic cellular changes, such as blebbing of the plasma membrane, cell shrinkage, DNA fragmentation, and chromosome condensation (O'Brien 1998). The programmed cell death process is induced in both invertebrates and vertebrates through different factors such as depletion of nutrients, survival factors from the culture media, and as a response to viral infection to prevent its replication and spread (O'Brien 1998). In cell culture, insect cells also undergo apoptosis as part of the antiviral defence when they are infected with a baculovirus. AcMNPV can enter and replicate in many insect species, as some researchers demonstrated that AcMNPV is unable to replicate in *S. littoralis* larvae (Rivkin et al. 2006). Whether AcMNPV infects *S. littoralis* is not well understood. So, we used this virus to examine if it can infect the newly established *S. littoralis* cell lines and then assess the mechanism of cell damage if replication occurred by AcMNPV and SpliMNPV.

Materials and methods

Virus strains and insect cell lines

AcMNPV and Sf21 cell lines were obtained from the Institute of Bioprocess Engineering, FAU-Erlangen-Nuremberg, Germany. SpliMNPV was kindly provided by Dr. David Grzywacz, University of Greenwich, UK. Spli-B and Spli-C cell lines were established in the cell culture laboratory of the above institute from the embryonic tissue of *S. littoralis* (the eggs of *S. littoralis* were obtained from Syngenta Company, Basel, Switzerland). The primary culture

was prepared from embryonic tissue in Hink's TNM-FH medium (Millipore Sigma, St. Louis, MO, USA), supplemented with 20% FBS, as the suspension was dispensed in 24-multiwell plates and incubated at 27 °C. After several subcultures, the cell lines were adapted to grow in suspension in serum free medium (Ahmed et al. 2014).

Virus stock preparation

The SpliMNPV inoculum was prepared from viral OBs, which were purified from infected larvae and suspended in ultrapure water (Ahmed et al. 2014). The virus stock was prepared from SpliMNPV OBs, according to Reid and Lua (2005) with some modification. The OBs were solubilized by incubation in alkaline solution (pH 11) at 27 °C until they lysed and then the solution was neutralized by insect medium with a final pH of 6.0–6.4. Subsequently, the virus solution was sterilized by filtration (0.22 µm) and stored at 4 °C. The *S. littoralis* cells were infected with ODV and the BV was harvested at around 3 days post-infection. Viral stock was prepared by infecting *S. littoralis* cells with BV at 0.1 multiplicity of infection (MOI) in 300-ml Erlenmeyer shaker flasks containing EX-cell 420 medium with a final culture volume of 50 ml for each flask at a cell density of 5×10^5 cells/ml. The cells were incubated at 27 °C and at 50 rpm in a shaker incubator. After 3–4 days post-viral inoculation, but before cell viability dropped to 85%, the virus was harvested by centrifugation at 180 g for 8 min to remove cells, while the supernatant containing virus particles was transferred to new 50 ml Falcon tubes and stored at 4 °C. For long-term storage, virus stocks were kept in insect medium, supplemented with 5% FBS and stored at – 80 °C.

Virus quantification

The virus titer was determined by endpoint dilution assay (Reed and Muench 1938). In this method, serial dilutions were prepared from the virus stock and each dilution was used to inoculate cells. The Spli-C cell line was used to measure SpliMNPV infectious viral particles, while the Sf21 cell line was used to estimate the AcMNPV titer. The number of infected cells was determined by examining cytopathic effects. One of the significant signs of viral infection, in addition to the cytopathic effects, is the formation of large OBs,

which can easily be observed under a light microscopy.

Testing the susceptibility of the *S. littoralis* cell lines to infection with AcMNPV and SpliMNPV

The susceptibility of the *S. littoralis* (Spli-B and Spli-C) cell lines to infection with AcMNPV and SpliMNPV was tested. Cells were seeded at a density of 5×10^5 cells/ml in a 6-well plate and infected with the virus. Thereafter, cells were incubated at 27 °C and cell cultures were checked daily for the cytopathic effects and OB formation to monitor the progress of infection. The two types of *S. littoralis* cell lines (Spli-B and Spli-C) and Sf21 were used to assess infection changes by AcMNPV. The cells were also seeded at a cell density of 5×10^5 cells/ml in EXCell420 medium in shaker flasks and infected with AcMNPV at MOI 1 and 5 PFU/cell. Cell density was determined each day using a haemocytometer chamber. To compare the ability of AcMNPV and SpliMNPV to replicate in the *S. littoralis* Spli-C cell line, the BV yields were determined by taking 1 ml aliquots from the cell culture after 48 and 72 h. The virus culture was stored at 4 °C until measuring the virus titer.

DNA fragmentation assay

DNA fragmentation assay was used to determine the induction of apoptosis in insect cells (Chejanovsky and Gershburg 1995). In these experiments, cells were infected with AcMNPV or SpliMNPV at MOI 1 PFU/cell. After 48 and 72 h post-infection, cells were harvested and the cell pellet was resuspended in 0.5 ml TES lysis buffer (10 mM Tris-base pH 8.0, 1 mM EDTA, and 1% SDS). Next, 100 ng/ml proteinase K (Millipore Sigma) was added and incubated at 37 °C for 2.5 h. Then, NaCl solution was added to the lysate and a final concentration of 1 M incubated overnight at 4 °C. Equal volumes of phenol:chloroform were added and mixed well by being inverted several times, and the mixture was centrifuged at 13,000 rpm for 3 min. The upper aqueous layer was transferred into a new tube, an equal volume of chloroform was added, and the mixture was inverted several times and centrifuged at 13,000 rpm for 3 min. The upper aqueous layer was transferred to a new tube, two volumes of ice-cold absolute ethanol was added, and the nucleic acid was precipitated by centrifugation at

13,000 rpm for 15 min. The nucleic acid pellet was resuspended in TE buffer containing RNaseA (20–50 µg/ml, Roche, Mannheim, Germany) and stored at –20 °C. The DNA samples were electrophoresed on 1.2% agarose gel.

Cell response to the viral infection

In order to observe cell changes after infection with AcMNPV and SpliMNPV in real time, cells were monitored with a video camera. The *S. littoralis* cells were seeded in a 24-well plate, which was placed onto the motorized stage of a high-end long-term imaging system (Nikon Eclipse Ti, Nikon, Japan) equipped with a cell culture incubator (Okolab, Napoli, Italy). Cells were cultured at 27 °C, and were imaged with a 10× objective (CFI Plan Fluor DL 10× Phase, N.A. 0.30, Nikon, Japan) for 72 h. Illumination from a xenon lamp (Lambda LS, Sutter Instruments, Novato, CA, USA), passing through a filter block was used to excite and detect fluorescence signal. Fluorescence was imaged by a sCMOS camera (NEO, Andor, Ireland) and digitized to disk onto a personal computer (Dell Precision T3500, Santa Clara, CA, USA) with Windows 7 operating System (Microsoft Corporation, Redmond, WA, USA). The primary resolution of the camera was 2560 × 2160 pixels; however, images were binned (2 × 2), resulting in a resolution of 1280 × 1080 pixels.

Results

Testing the susceptibility of the *S. littoralis* cell lines to infection with AcMNPV and SpliMNPV

The SpliMNPV was successfully infected and replicated in both *S. littoralis* cell lines. The cytopathic effects, such as hypertrophy of nuclei, slow cell growth and large size, were observed post-infection. Viral OBs were also observed inside the cell nuclei after 48–72 h, and with time, many more OBs were produced in the cells. A few days post-infection, some of the infected cells were lysed and OBs were released into the culture medium (Fig. 1). The results showed that the Spli-C cell line produces about seven fold higher than Spli-B (data not shown).

The microscopic observation of the *S. littoralis* cells that were infected with AcMNPV showed cell

Fig. 1 Light microscopic observations of uninfected and infected *S. littoralis* cells. **a** Uninfected cells, **b** cells infected with SpliMNPV showing the appearance of OBs inside the cells, **c** cells infected with AcMNPV

membrane blebbing, forming apoptotic bodies (Fig. 1). The vast majority of the cells lost their viability and stained with propidium iodide (PI) after less than 24 h post-infection with AcMNPV, while uninfected and infected cells with SpliMNPV remained viable (Fig. 2). Only a few OBs were observed in AcMNPV infected cells, where most of the cells died after a few hours post-infection.

Monitoring *S. littoralis* response to AcMNPV and SpliMNPV using a video camera showed that the infected cells with AcMNPV underwent dramatic changes in morphology and formed small bodies that led to cell death (Online Resource 2-Fig. S2) when compared to uninfected cells (Online Resource 1-Fig. S1). Moreover, cells infected with SpliMNPV remained viable while many viral OBs were produced (Online Resource 3-Fig. S3).

S. littoralis cell lines, which were infected with AcMNPV, accumulated as composed balls in

suspension culture in shaker flasks a few hours after infection, while there was no cell clumping noticed in the uninfected cells or those infected with SpliMNPV. Conversely, infected and uninfected Sf21 cells with AcMNPV did not form any ball accumulations (Fig. 3). The density of Sf21 cells infected with AcMNPV at MOI 1 PFU/cell decreased 2 days post-infection and 1 day post-infection when MOI 5 PFU/cell was used. The decrease in cell densities remained constant with time, compared to uninfected cells. On the other hand, the cell densities of the Spli-B and Spli-C cells that were infected with AcMNPV at 1 and 5 MOI showed a considerable decrease after a few hours post-infection compared to uninfected cells.

AcMNPV and SpliMNPV virus concentrations

AcMNPV BVs, produced by Spli-C cells, were very low compared to SpliMNPV. The titer of the

Fig. 2 Light micrographs of uninfected and infected Spli-C cells with SpliMNPV and AcMNPV (magnification $\times 200$). **a** Uninfected cells, **b** uninfected cells stained with propidium

iodide (PI), **c** cells infected with SpliMNPV, **d** cells infected with SpliMNPV and stained with PI, **e** cells infected with AcMNPV, **f** cells infected with AcMNPV and stained with PI

AcMNPV BVs was 3.2×10^4 and 2.6×10^4 (PFU/ml) after 48 and 72 h, respectively. Conversely, the SpliMNPV titer was $1\text{--}3 \times 10^7$ (PFU/ml) after 48 h and increased to 6.5×10^7 (PFU/ml) after 72 h.

DNA fragmentation assay

Spli-C cells were infected with AcMNPV at MOI 1 PFU/cell and the total cellular DNA was extracted

Fig. 3 *Spodoptera littoralis* cell growth in shaker flasks. **a** Cells infected with SpliMNPV, **b** cells infected with AcMNPV

from 5 to 6×10^6 cells, 48 and 72 h post-infection. SpliMNPV was also used in these experiments for comparison. The oligonucleosome ladders were observed by using DNA electrophoresis in Spli-C cells infected with AcMNPV. At the same time, there was no DNA fragmentation observed in the Spli-C cells infected with SpliMNPV. DNA fragmentation was absent in uninfected Sf21 cell lines or in cells infected with AcMNPV, where there were no oligonucleosome ladders detected on an agarose gel (Fig. 4).

Discussion

The newly established *S. littoralis* cell lines were tested to observe if the cells could support viral replication and OB production. Some studies indicated that *S. littoralis* larvae were quite resistant to oral AcMNPV infection. Yet, *S. littoralis* caterpillars were sensitive to BV infection when BV was injected into caterpillar haemolymph (Rivkin et al. 2006). The

researchers concluded that the *S. littoralis* larvae resistance to AcMNPV resulted from association of humoral and cellular immune responses. It reacts to the AcMNPV infection by encapsulating and melanising the infected tracheal cells (Rivkin et al. 2006). Results of this study revealed that *S. littoralis* cells were resistant to AcMNPV infection in vitro.

Also, the microscopic examination showed that the *S. littoralis* cells infected with AcMNPV exhibited morphological changes, such as cells breaking into small bodies a few hours post-infection. Most of the infected cells with AcMNPV were stained with propidium iodide (PI), which can penetrate only dead cells. It is used for the detection of dead cells, as well as for the differentiation between apoptotic and normal cells (Boutonnat et al. 2000; Mazzini et al. 2003). Our results also demonstrated that infected cells with SpliMNPV remained viable. Therefore, the cell membrane remained active and excluded the PI stain.

Fig. 4 Cellular DNA fragmentation analysis on a 1.2% agarose stained with propidium iodide (PI). **a** Lane M: DNA size II, lane 1: Spli-C non-infected, lane 2: Spli-C infected with SpliMNPV, lane 3: Spli-C cells infected with AcMNPV, lane 4: Sf21 non-

infected and lane 5: Sf21 infected with AcMNPV. **b** Lane M: DNA size II, lane 1: Spli-C infected with SpliMNPV, and lane 2: Spli-C infected with AcMNPV

The *S. littoralis* cells infected with AcMNPV also showed changes in their DNA, where the DNA ladder was observed on the agarose gel. The DNA ladders are normally formed as a result of DNA degradation by endonucleases to small oligonucleosome fragments during cell apoptosis (Lai et al. 2006).

The attached movies clearly show how the *S. littoralis* cells responded to the infection with AcMNPV and SpliMNPV. In suspension culture, the *S. littoralis* cells infected with AcMNPV were composed balls that accumulated a few hours after infection, with cell density decreasing dramatically. The cells aggregated due to a response to viral infection, which led to significant reduction in the virus titer, compared to that seen with SpliMNPV infections. Another study found that *S. littoralis* cells (SL2) infected with AcMNPV produced a very low BV and polyhedrin compared with *S. frugiperda* infected with the same virus. The researchers also observed cell blebbing, culminating in post-viral infection. After conducting DNA fragmentation assay and fluorescent dye DAPI staining, it was concluded that the *S. littoralis* cells underwent apoptosis post-infection with AcMNPV (Chejanovsky and Gershburg 1995). The host-viral interplay may prevent viral replication and spread using different mechanisms, yet at the same time, the virus' improved strategies to survive evaded host defences (Clem and Miller 1993).

One of the essential host defence mechanisms is apoptosis, in which a group of enzymes called caspases, play a central role in it, which include two types: initiator and effector. A variety of signals activate initiator caspases that in turn activate effector caspases. The activated effector caspases cleave cellular proteins, leading to apoptotic cell death. Caspase-1 is the main effector caspase in the *S. littoralis* insect (Liu et al. 2005; Guy and Friesen 2008; Richard and Tulasne 2012).

Studies on baculovirus gene expression in insect non-permissive cell lines indicate that each group had its own mechanism to interact with viral promoters (Morris and Miller 1992). One is that the baculovirus must inhibit cell apoptosis to be able to survive and multiply; a group of genes also play a role as anti-apoptotic proteins: p35, p49, and IAPs (Seshagiri and Miller 1997; Clem 2001; Brand et al. 2011). Previous studies show that the viral anti-apoptotic proteins play an essential role in controlling host apoptosis (Brand et al. 2011). The viral anti-apoptotic proteins in the case of AcMNPV could be expressed late, such that the insect cells had already started the apoptosis process. Alternatively, the level of gene expression was not high enough to stop apoptosis.

Antiviral RNAi is another host defence mechanism generated by the insect against the baculovirus, in order to limit virus replication and to attenuate the

viral infection. However, the baculovirus has developed viral suppressors (VSRs) to fight the host RNAi (Mehrabadi et al. 2015). Several other studies have been done to understand the baculovirus host interaction at the mRNA level, but the exact mechanisms of the insect host response to the baculovirus is not fully understood (Nguyen et al. 2013a, b). Further studies are required to provide answers to the host antiviral defence, which could manipulate the host to increase virus particles or recombinant protein production per cell (Nguyen et al. 2013a, b). The recent development in OMIC technologies can play a key role in discovering significant molecules, which have a similar role in the intracellular interactions between viruses and their hosts (Berkhout and Coombs 2013; Shrestha et al. 2018).

In conclusion, the newly established embryonic *S. littoralis* cell lines showed high resistance to AcMNPV by undergoing apoptosis, which play a significant role in preventing viral replication and viral OBs production. On the other hand, SpliMNPV was able to evade the host barriers and succeed in replicating and producing a number of viral OBs.

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