

An *Anticarsia gemmatalis* multiple nucleopolyhedrovirus mutant, vApAg, induces hemocytes apoptosis in vivo and displays reduced infectivity in larvae of *Anticarsia gemmatalis* (Hübner) (Lepidoptera: Noctuidae)

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Received 20 March 2007; received in revised form 4 June 2007; accepted 11 June 2007

Available online 23 July 2007

Abstract

An *Anticarsia gemmatalis* multiple nucleopolyhedrovirus (AgMNPV) mutant, vApAg, induces apoptosis in a cell culture derived from *Anticarsia gemmatalis* (UFL-AG-286), reducing viral progeny. We have investigated apoptosis induction in vivo by vApAg in *A. gemmatalis* larvae and its correlation to infectivity reduction. LC₅₀, LD₅₀, LT₅₀ and the mean time to death of larvae were determined for vApAg and AgMNPV. Apoptosis was assessed for hemocytes of infected larvae using light and transmission electron microscopy. All types of hemocytes can be infected by vApAg. After 12 h post-infection (h p.i.), typical cellular modifications associated to nucleopolyhedrovirus infection were observed. Apoptosis becomes evident after 24 h p.i., and massive after 72 h p.i. Necrosis of infected cells was also observed. Despite cell death, hemocytes produced budded viruses and polyhedra. Pl and gh1-type hemocytes presented phagocytic activity. Agarose gel electrophoresis revealed fragmentation of hemocytes DNA at late times post-infection. The LC₅₀ and LD₅₀ were between five- and six-fold higher for vApAg. The LT₅₀ and the mean time to death were higher for vApAg in a same treatment or for a similar mortality induced by AgMNPV. These results show correlation of apoptosis and the reduced infectivity of vApAg in *A. gemmatalis* larvae.

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Keywords: Apoptosis; Baculovirus; Hemocytes; Infectivity reduction; Lepidoptera; Ultrastructure

1. Introduction

Apoptosis is a kind of programmed cell death conserved among metazoans, playing important functions in immunity, development, cell differentiation and tissue homeostasis. Apoptosis can be triggered by diverse stimuli, which results in cysteine

protease activation that drives cell shrinkage, DNA cleavage by endonucleases, and cell fragmentation into apoptotic bodies (Ashe and Berry, 2003; Kerr et al., 1972).

Apoptosis regulation by members of the family *Baculoviridae* has been extensively studied since the beginning of the 1990s, when a mutant of *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) was shown to induce apoptosis in a cell line derived from *Spodoptera frugiperda* (Clem et al., 1991).

Two types of apoptosis inhibitors are described for baculoviruses: P35 protein and inhibitor of apoptosis (IAP) proteins. P35 is a broad-spectrum caspase inhibitor present in baculoviruses and more recently found in entomopoxviruses, while IAP constitutes a protein family whose members are described in baculoviruses, insects, nematodes and humans (Clarke and Clem, 2003a; Clem, 2001; Means et al., 2007).

Mutation in *p35* and *iap* or the impairment of their expression, results in premature death and reduction in viral progeny

Abbreviations: AcMNPV, *Autographa californica* multiple nucleopolyhedrovirus; AgMNPV, *Anticarsia gemmatalis* multiple nucleopolyhedrovirus; BV, budded virus; DIC, differential interferential contrast; gh1, granular hemocyte type 1; gh2, granular hemocyte type 2; h p.i., hours post-infection; IAP, inhibitor of apoptosis; LC₅₀, lethal concentration to kill 50% of individuals in a bioassay; LD₅₀, lethal dose to kill 50% of individuals in a bioassay; PFU, plaque forming unit; PIBs, polyhedral inclusion bodies; pl, plasmatocyte; pr, prohemocyte; sph, spherulocyte; TEM, transmission electron microscopy

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production in specific hosts, as demonstrated mainly for AcMNPV and *Bombyx mori nucleopolyhedrovirus* (BmNPV) (Clarke and Clem, 2003a; Clem, 2001). In other cases, despite presenting intact anti-apoptotic genes, baculoviruses like AcMNPV induce apoptosis and display reduced infectivity and propagation in a specific host, as *Spodoptera litura* larvae (Zhang et al., 2002). Wild-type *Heliothis armigera single nucleopolyhedrovirus* (HaSNPV) induces apoptosis in a cell line derived from *Trichoplusia ni* (Dai et al., 1999). This demonstrates that putative viral strategies to counteract apoptosis are one of the determinants of baculovirus host range.

Although it has long been reported that baculoviruses lacking an anti-apoptotic gene have a reduced infectivity in vivo, to date, there are only three reports concerning baculovirus induction of apoptosis in lepidopteran larvae (Clarke and Clem, 2003b; Silveira et al., 2005; Zhang et al., 2002). Studies in this field allowed the recognition of apoptosis as an important anti-viral response in insects, which lack acquired immunity elements such as antibodies, for example (Clarke and Clem, 2003a; Clem, 2001).

The baculovirus *Anticarsia gemmatalis multiple nucleopolyhedrovirus* (AgMNPV) has been used for more than 20 years in Brazil to control the velvetbean caterpillar, *Anticarsia gemmatalis* (Hübner). It represents an important factor for the reduction in production costs and environmental damages caused by chemical pesticides in soybean (*Glycine max*) crops (Moscardi, 1999; Ribeiro et al., 1998). AgMNPV is a rod-shaped, enveloped virus with circular, double-stranded, 132,239 bp DNA genome, pathogenic to lepidopterans, and belongs to the NPV I group (Grasela and McIntosh, 1998; Johnson and Maruniak, 1989; Oliveira et al., 2006).

During the construction of a recombinant baculovirus derived from AgMNPV, besides recombinant and non-recombinant viruses, a mutant, vApAg, was obtained. This mutant induces premature death in a cell line highly permissive to AgMNPV (UFL-AG-286) (Sieburth and Maruniak, 1988a) while, in another cell line (BTI-Tn-5B1-4) (Tn-5B) (Granados et al., 1994), it replicates normally. UFL-AG-286 cells infected by vApAg die by apoptosis (Silveira et al., 1999). Despite apoptosis occurrence, budded viruses (BV) and polyhedral inclusion bodies (PIBs) are produced (Silveira et al., 1999), but in a reduced fashion if compared with the progeny obtained by the wild-type virus in the same cell line (Castro and Ribeiro, 2001).

AgMNPV is known to possess at least one type of *iap* gene related to apoptosis inhibition (Carpes et al., 2005) (GenBank accession no. AY525121). The gene *iap-3* was shown to be disrupted by a transposable element in the vApAg virus (Carpes et al., unpublished), which may be the cause of apoptosis induction in cell culture and in *A. gemmatalis* larvae.

In this work, we have investigated if the vApAg mutant induces apoptosis in vivo by accessing vApAg infection in *A. gemmatalis* hemocytes by intrahaemocoelic inoculation. Light and transmission electron microscopy were used to follow the temporal events of infection in hemocytes. Agarose gel electrophoresis was conducted to detect oligonucleosomal fragmentation of hemocytes' DNA. The infectivity was compared between vApAg and AgMNPV by the determination of the mean

lethal dose (LD₅₀) for intrahaemocoelic infection, the mean lethal concentration (LC₅₀) for oral infection, the mean lethal time (LT₅₀) and the mean time to death for both.

2. Materials and methods

2.1. Insects and viruses

A. gemmatalis eggs were provided by Embrapa Recursos Genéticos e Biotecnologia and Embrapa Soja, and reared on artificial diet (Greene et al., 1976) at 27–28 °C, with a 12:12 h dark/light regime. The molts were monitored, and fourth instar larvae (between 0 and 24 h after molt) were used for all experiments.

AgMNPV isolate 2D (Grasela and McIntosh, 1998) was propagated in UFL-AG-286 cells (Grasela and McIntosh, 1998; Sieburth and Maruniak, 1988b). VApAg was propagated in BTI-Tn-5B1-4 (Tn-5B) cells (Granados et al., 1994). Cell cultures were maintained in TC-100 medium (GIBCO-BRL Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum at 27 °C. Virus inocula were titered by the TCID₅₀ method, following the protocol described by O'Reilly et al. (1992).

AgMNPV-2D and vApAg (PIBs) were obtained from *A. gemmatalis* larvae, which died by infection with the respective virus. The cadavers were macerated in homogenization buffer and filtered. The filtrate was centrifuged, the pellet resuspended in distilled water and maintained at –20 °C.

2.2. Bioassays

Insects were injected with 10 µl of viral inoculum directly into the haemocoel, by using an insulin micro-syringe. Six different doses were used for AgMNPV (0.01, 0.05, 0.1, 1, 10 and 100 PFU) and five for vApAg (0.1, 1, 5, 10 and 100 PFU). Controls were obtained by the inoculation of an equal volume of TC-100 medium (mock-infected). For each treatment, 50 larvae were inoculated. Each larva was reared separate in a plastic cup.

For oral infection, purified PIBs were used in five concentrations for both viruses (5×10^2 , 1.5×10^3 , 4.5×10^3 , 1.35×10^4 , 4.05×10^4 PIBs/ml). For each treatment 30 larvae were used, which were maintained in plastic cups in groups of three individuals per cup. Each cup contained 5 ml of artificial diet, free of nipagin and formol, covered by 150 µl of viral inoculum. For controls, distilled water was used instead of viral inoculum.

Development and mortality were analyzed every day up to the end of the experiment. Individuals that pupated were considered to have survived the infection. Deaths attributed to inoculation trauma were not considered. Larvae were considered dead if they did not move after mechanical stimulation. A sample of hemolymph was taken from dead larvae to confirm baculovirus infection by finding PIBs under light microscopy.

Mortality data were submitted to Probit analysis (Finney, 1971) to estimate the LD₅₀ for intrahaemocoelic infection, the LC₅₀ for oral infection, and the LT₅₀ for specific doses/concentrations for both ways of infection, as well associated parameters (95% fiducial limit, slope, χ^2) for both viruses.

For statistical significance between AgMNPV and vApAg, regarding the LD₅₀, LC₅₀ and LT₅₀ values, the non-overlapping of the 95% fiducial limits was considered as evidence of significant differences. The mean time to death was determined according to Morales et al. (2001).

2.3. Light and transmission electron microscopy

Hemolymph samples of insects infected intrahaemocoelically with 20 µl of vApAg inoculum (10⁸ PFU/ml), as described above, were collected in anticoagulant buffer (98 mM NaOH, 186 mM NaCl, 1.7 mM EDTA, 41 mM citric acid, pH 4.5) (Mead et al., 1986) after different times post-infection (12, 24, 48 and 72 h p.i.) and observed under differential interferential contrast (DIC) in an Axiophot Zeiss light microscope. For transmission electron microscopy, the samples were fixed for 30 min (2% glutaraldehyde, 2% paraformaldehyde in 0.1 M sodium cacodylate buffer pH 7.4 with 5% sucrose), centrifuged at 750 × g for 5 min, the pellet washed in the same buffer, post-fixed (1% osmium tetroxide, 0.8% potassium ferricyanide in the same buffer), contrasted in block with 0.5% uranyl acetate, dehydrated in acetone, and embedded in Spurr's resin. The ultrathin sections were contrasted with uranyl acetate/lead citrate and observed in a TEM JEOL 100C and JEOL 1011 at 80 kV.

2.4. Hemocyte identification

Larval *A. gemmatalis* hemocyte types were identified by structural and ultrastructural characteristics described previously (Silveira et al., 2003, 2004).

2.5. DNA extraction and oligonucleosomal fragmentation assay

Hemolymph samples obtained from insects inoculated with 20 µl of viral inoculum (10⁸ PFU/ml), as described above, were collected in PBS pH 7.2 at 12, 24, 48 and 72 h p.i., and submitted to DNA extraction according to Aljanabi and Martinez (1997). In brief, samples were centrifuged at 1300 × g for 6 min, the pellet resuspended in 400 µl lysis buffer (0.4 M NaCl, 10 mM Tris-HCl pH 8.0, 2 mM EDTA pH 8.0), 1 mg/ml proteinase K, 2% SDS and incubated at 50 °C for 1 h. Then, 400 µl of 6 M NaCl were added, the tubes were gently vortexed and centrifuged at 16,000 × g for 20 min. Equal volume of isopropanol was added to the supernatant, it was homogenized and maintained

Table 1

Mean lethal dose (LD₅₀), 95% fiducial limits of the LD₅₀ (95% FL), slope and Chi-square (χ²) values for *Anticarsia gemmatalis* larvae infected with AgMNPV and vApAg by intrahaemocoelic injection of BVs

Virus	LD ₅₀ ^a	95% FL ^a	Slope	χ ²
AgMNPV	0.744	0.41–1.54	0.96	2.29 ns
vApAg	4.170	2.85–6.61	1.46	2.24 ns

^a Expresses the number of PFU/larva. Non-overlapping 95% FL is an evidence of significant differences between the LD₅₀ values obtained.

Table 2

Mean lethal time (LT₅₀), 95% fiducial limits of the LT₅₀ (95% FL), slope and Chi-square (χ²) values for *A. gemmatalis* larvae infected with LD₅₀ higher doses of AgMNPV and vApAg BVs by intrahaemocoelic injection

Virus	Dose (PFU/larva)	LT ₅₀ ^a	95% FL ^a	Slope	χ ²
AgMNPV	1	9.75	8.98–11.06	5.83	3.41 ns
	100	5.72	5.47–6.00	11.45	7.72 ns
vApAg	5	12.12	11.31–13.69	7.53	2.76 ns
	100	9.1	8.7–9.48	9.20	1.31 ns

^a Expresses time in days. Non-overlapping 95% FL is an evidence of significant differences between the LT₅₀ values obtained.

at –20 °C for at least 1 h. The DNA was precipitated by centrifugation at 16,000 × g for 20 min, washed with 70% ethanol, dried and resuspended in 50 µl TE (10 mM Tris-HCl, 1 mM EDTA pH 8.0). Ribonuclease A was added to a final concentration of 40 µg/ml. DNA concentration was estimated according to phage λ standards in 0.8% agarose gel electrophoresis. Approximately 2 µg of each sample were analyzed in a 3% agarose gel at 80 V and visualized under UV radiation after incubation with ethidium bromide.

3. Results

3.1. Bioassays

For intrahaemocoelic infection, it was obtained LD₅₀ values of 0.744 and 4.17 PFU/larva for AgMNPV and vApAg, respectively, which means that the mutant vApAg requires around a six-fold higher dose than the wild-type virus to kill 50% of larvae in a bioassay (Table 1). The LT₅₀ (Table 2) values obtained for doses that result in, approximately, 50 and 100% of mortality show that vApAg requires between two and three more days than AgMNPV to kill 50% of larvae when using doses that result in similar mortalities for both viruses. The mean time to death

Table 3

Mortality and mean time to death of *A. gemmatalis* larvae intrahaemocoelically infected with AgMNPV and vApAg BVs

Virus	Dose (PFU/larva)	Number of dead larvae ^a	n ^a	Mortality (%) ^a	Mean time to death (days) ^a
AgMNPV	0.1	10.33 ± 0.58	42 ± 1	24.60 ± 0.90	8.39 ± 1.85
	1	20 ± 3.61	38.67 ± 6.81	51.91 ± 4.82	7.14 ± 0.93
	100	36.67 ± 4.04	38 ± 3.46	96.43 ± 4.29	5.87 ± 0.40
vApAg	0.1	2 ± 2.65	36.33 ± 4.16	4.86 ± 5.86	10.60 ± 0.57
	1	13 ± 3	43 ± 3	30.51 ± 8.59	10.95 ± 2.45
	100	40.67 ± 7.37	41.67 ± 6.66	97.41 ± 2.51	9.33 ± 2.37

^a Values correspond to media and standard deviation for three repetitions. Deaths attributed to injection trauma were not considered, causing variable n.

Table 4

Mean lethal concentration (LC₅₀), 95% fiducial limits of the LC₅₀ (95% FL), slope and Chi-square (χ^2) values for *A. gemmatilis* larvae orally infected with AgMNPV and vApAg

Virus	LC ₅₀ ^a	95% FL ^a	Slope	χ^2
AgMNPV	723.10	185.92–1382.64	1.86	5.83
vApAg	3718.07	1686.91–7377.35	0.75	4.72

^a Expresses the number of PIBs/ml. Non-overlapping 95% FL is an evidence of significant differences between the LC₅₀ values obtained.

Table 5

Mean lethal time (LT₅₀), 95% fiducial limits of the LT₅₀ (95% FL), slope and Chi-square (χ^2) values for *A. gemmatilis* larvae orally infected with LC₅₀ higher doses of AgMNPV and vApAg PIBs

Virus	Concentration (PIBs/ml)	LT ₅₀ ^a	95% FL ^a	Slope	χ^2
AgMNPV	4,500	8.30	7.64–8.89	6.70	6.47
	13,500	7.40	6.65–8.01	5.57	3.77
	40,500	5.91	4.88–6.57	6.99	7.14
vApAg	4,500	14.08	12.44–17.55	6.34	21.18
	13,500	10.63	9.95–11.44	5.41	8.24
	40,500	8.83	8.24–9.50	4.90	3.27

^a Expresses time in days. Non-overlapping 95% FL is an evidence of significant differences between the LT₅₀ values obtained.

(Table 3) values reveal that vApAg requires between two and four more days than AgMNPV to kill larvae using the same dose.

For oral infection, the LC₅₀ values obtained were 723.1 and 3718.07 PIBs/ml for AgMNPV and vApAg, respectively, which reveals that a five-fold higher concentration is needed for the mutant virus to kill 50% of larvae in a bioassay (Table 4). The LT₅₀ values presented significant differences between the viruses for the same concentration, showing that vApAg requires between three and six more days to kill 50% of larvae in comparison to AgMNPV (Table 5). For a similar LT₅₀ (around 8 days), vApAg requires a 9-fold higher concentration than AgMNPV (Table 5). For the same dose, the mean time to death (Table 6) values reveal that vApAg requires between one and two more days than AgMNPV to kill larvae.

In comparison to AgMNPV, vApAg produced diminished larval mortality for all the concentrations tested, for both, intrahaemocoelic and oral infection (Tables 3 and 6). For intrahaemocoelic infection, a maximum reduction of approximately 80% was obtained for 0.1 PFU/larvae (Table 3). For oral infection, the maximum reduction was around 40%, obtained for 4500 PIBs/ml (Table 6).

Table 6

Mortality and mean time to death of *A. gemmatilis* larvae orally infected with AgMNPV and vApAg PIBs

Virus	Concentration (PIBs/ml)	Number of dead larvae ^a	Mortality (%) ^a	Mean time to death (days) ^a
AgMNPV	4,500	26.33 ± 3.21	87.77 ± 10.72	8.56 ± 0.65
	13,500	27.67 ± 1.53	92.22 ± 5.09	8.13 ± 0.56
	40,500	28.33 ± 0.58	94.44 ± 1.93	6.98 ± 0.12
vApAg	4,500	14.67 ± 4.04	48.89 ± 13.48	9.45 ± 0.22
	13,500	20.00 ± 2.00	66.67 ± 6.67	8.93 ± 0.57
	40,500	24.33 ± 1.53	81.11 ± 5.09	8.63 ± 0.25

^a Values correspond to media and standard deviation for three repetitions. *N* = 30.

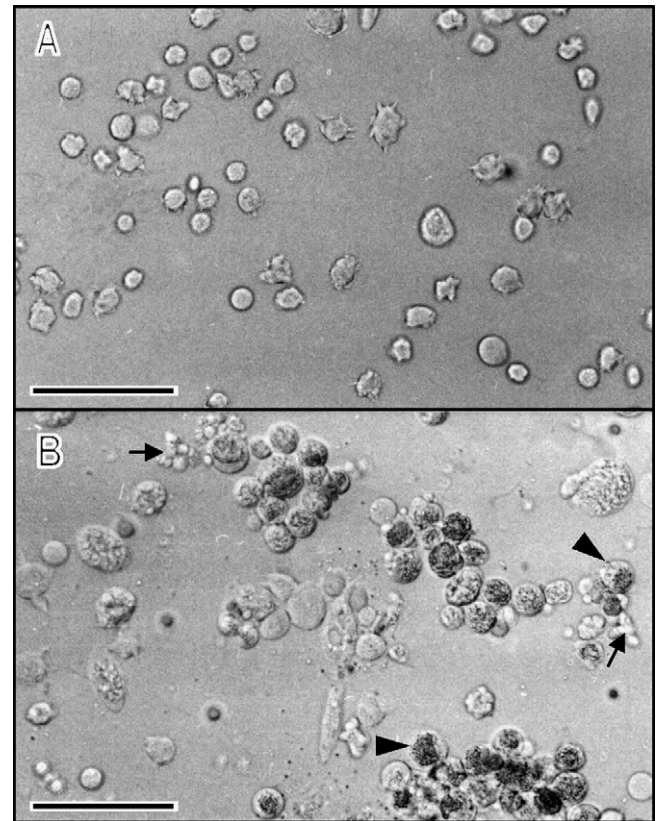


Fig. 1. DIC micrographs of *Anticarsia gemmatilis* larval hemocytes infected with vApAg: (A) 12 h p.i.; (B) 72 h p.i. Arrowheads, cells presenting PIBs and arrows, apoptotic bodies. Bars represent 65 μ m.

3.2. Light and transmission electron microscopy (TEM)

Six hemocyte types were recognized in *A. gemmatilis* larvae based on structural and ultrastructural characteristics, as described previously (Silveira et al., 2003, 2004). They were prohemocytes (pr), plasmatocytes (pl), granular hemocytes type 1 (gh1), granular hemocytes type 2 (gh2), oenocytoids (oe), and spherulocytes (sph). The ultrastructure described originally for each cell type was observed in controls (not shown).

By DIC, the hemocytes morphology was similar to control, after 12 and 24 h p.i. with a small number of fragmented cells (Fig. 1A). By the TEM, between 12 and 24 h p.i. (Fig. 2A–D) nuclei were hypertrophied, with a slight folding of the nuclear envelope. The heterochromatin was margined

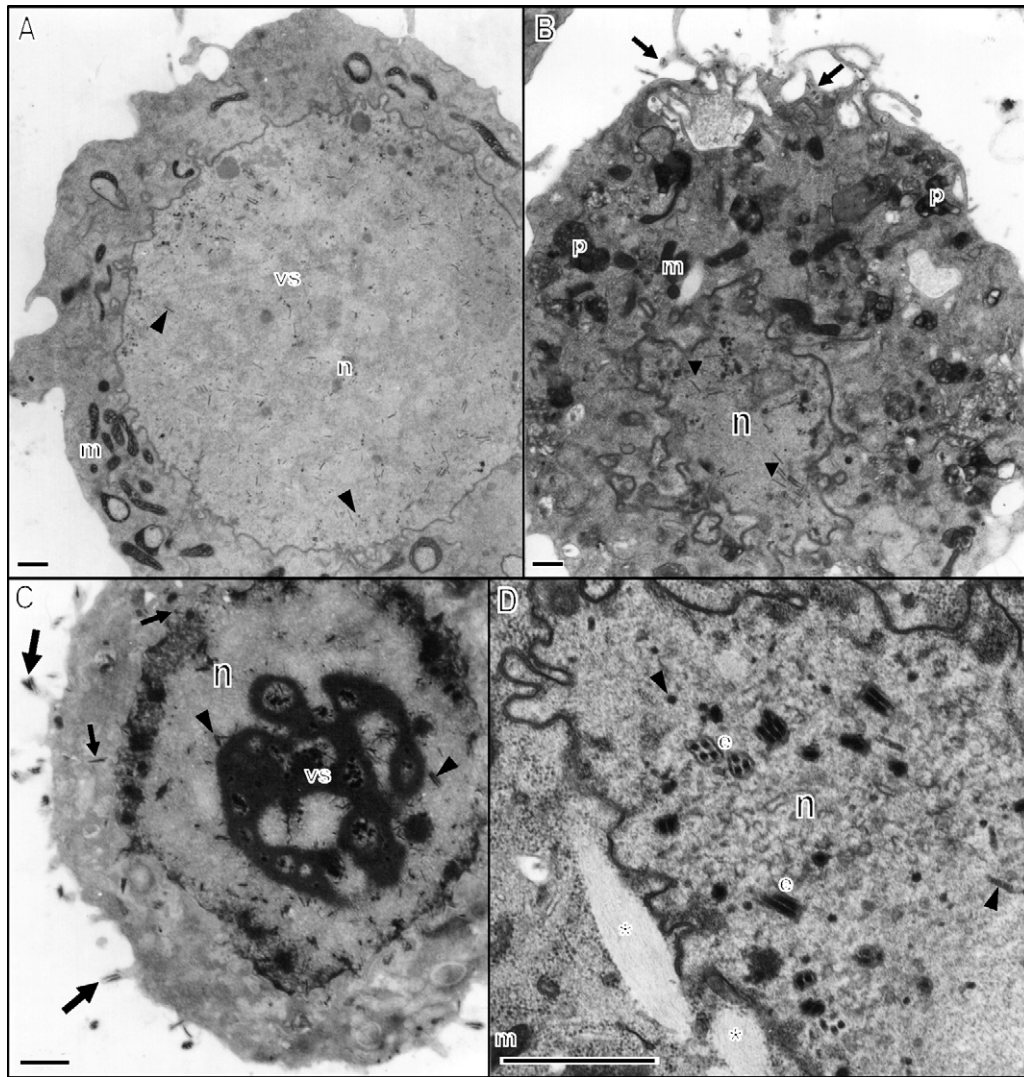


Fig. 2. Transmission electron micrographs of *A. gemmatilis* larval hemocytes after 12 and 24 h p.i. with vApAg. (A) 12 h p.i. Oenocitoid, presenting a hypertrophied nucleus (n) containing an immature virogenic stroma (vs). Arrowheads, nucleocapsids; n, nucleus and m, mitochondria. (B) 12 h p.i. Granular hemocyte type 1 presenting some BVs budding at the plasma membrane (arrows) and phagolysosomes (p). Arrowheads, nucleocapsids; n, nucleus and m, mitochondria. (C) 24 h p.i. Putative prohemocyte displaying a mature virogenic stroma (vs), nucleocapsids transportation through the cytoplasm (small arrows) and intense BVs budding at the plasma membrane (large arrows). Arrowheads, nucleocapsids; n, nucleus. (D) 24 h p.i. Plasmatocyte displaying nucleocapsids envelopment by membranous profiles (e) and cytoplasmic fibrillar aggregates (*). Arrowheads, nucleocapsids; n, nucleus and m, mitochondria. Bars represent 1 μ m.

and the virogenic stroma became evident, presenting nucleocapsids assembling. In the nuclear periphery, nucleocapsids were enveloped by membranous profiles. In this period, fibrillar aggregates appeared and accumulated in the cytoplasm (Fig. 2D). Nucleocapsids were directed to the cytoplasm by budding through the nuclear envelope, and BVs were seen to protrude at the plasma membrane (Fig. 2B and C). Chromatin condensation was observed for some cells (not shown).

After 48 and 72 h, an extensive number of fragmented cells and PIBs could be observed by DIC (Fig. 1B). By TEM, PIBs assembling, extensive nuclear and cytoplasmic fibrillar aggregates were visualized (Figs. 3A and B, 4A–C). For some cells, the nuclear envelope was greatly expanded, forming a convoluted network through the adjacent cytoplasm (Fig. 4A). These expanded nuclei were sometimes shown to be broken, causing the mix of nuclear and cytoplasmic compartments, with the

presence of PIBs and groups of unenveloped nucleocapsids just beneath the plasma membrane (Fig. 4B and C).

Endomembranes dilation, plasma membrane blebbing and cell fragmentation into apoptotic bodies were observed at 12 h p.i., however, they became more frequent at late times post-infection. Apoptosis was observed for cells that did not present viral cytopathic effects, and for infected cells in different phases of viral morphogenesis (Fig. 5A–E). The pattern of cell fragmentation was shown to be variable (Fig. 5A–D). BV entry and intense budding were observed for apoptotic cells or apoptotic bodies (Fig. 5B, D and E). It was not possible to detect production of apoptotic bodies for sph, despite their evident infection (Figs. 3B and 7A).

Phagocytic activity was observed at 12 h p.i. for pl and gh1 (Fig. 2B) and like apoptosis events, it became more intense at late times post-infection. These two hemocyte types were observed

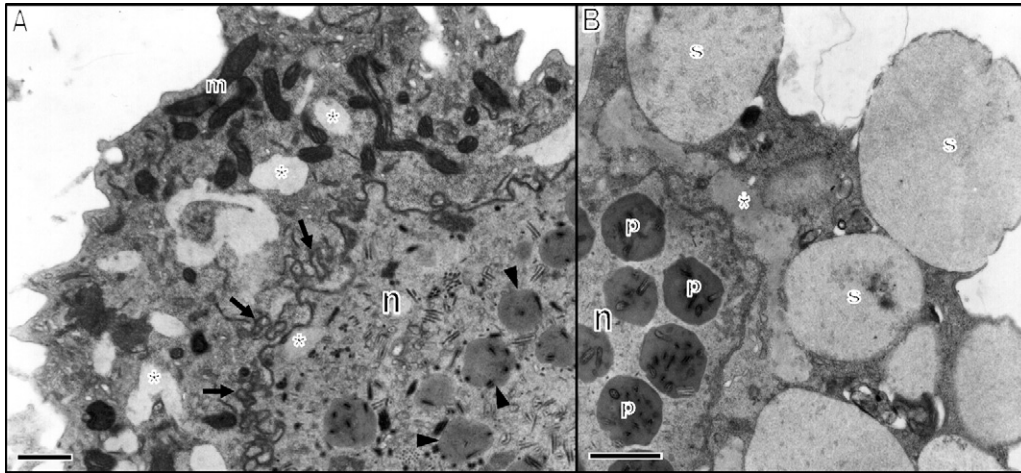


Fig. 3. Transmission electron micrographs of *A. gemmatalis* larval hemocytes after 48 and 72 h p.i. with vApAg. (A) 72 h p.i. plasmatocyte presenting nuclear and cytoplasmic fibrillar aggregates (*), nuclear envelope folding (arrows) and PIBs assembling (arrowheads); n, nucleus and m, mitochondria. (B) 48 h p.i. spherulocyte; n, nucleus; p, PIBs; s, spherule and (*), fibrillar aggregate. Bars represent 1.5 μm .

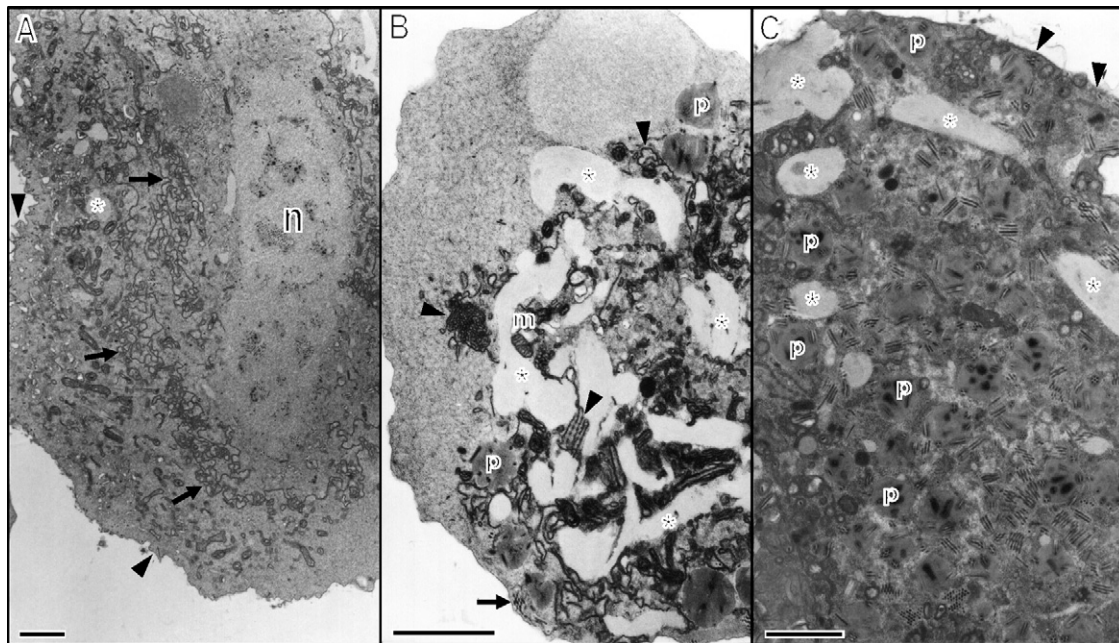


Fig. 4. Transmission electron micrographs of *A. gemmatalis* larval hemocytes after 48 and 72 h p.i. with vApAg, which presented cytoplasmic disorganization. (A) 48 h p.i. unidentified hemocyte. Note the intensely extended nuclear envelope (arrows), and the numerous nucleocapsids in the periphery of the cytoplasm (arrowheads); n, nucleus and (*), fibrillar aggregate. (B) 72 h p.i. unidentified hemocyte. The nucleus was broken and remnants of the nuclear envelope (arrowheads) were mixed with fibrillar aggregates (*), nucleocapsids (arrows), mitochondria (m) and PIBs (p). (C) 72 h p.i. unidentified hemocyte. A great number of PIBs (p) were produced, the nuclear envelope was ruptured, some PIBs (p) and free nucleocapsids (arrowheads) lie beneath the plasma membrane. (*) Fibrillar aggregates. Bars represent 2 μm .

to phagocytose apoptotic bodies, entire cells and free PIBs, but the engulfment of large bodies, such as entire cells, was more common for pl (Fig. 6A–C). Necrosis of infected cells, presenting a mature virogenic stroma or even PIBs, was frequently observed (Fig. 7A and B). All hemocyte types were shown to be infected, but for gh2, this occurred in an extremely low frequency.

3.3. DNA oligonucleosomal fragmentation assay

Agarose gel electrophoresis of hemocytes DNA revealed that for mock- (not shown) and AgMNPV-infected insects, there was

no DNA fragmentation (Fig. 8A). For vApAg, ladders were not visualized, but smears were found at 48 and 72 h p.i., periods when apoptosis was more evident (Fig. 8B).

4. Discussion

By studying baculovirus interactions with their hosts, intriguing questions can be elucidated related to the specificity of infection, such as the diversity of survival strategies developed by both viruses and insects over millions of years of coevolution. In this scenario, the knowledge about apoptosis as a defense

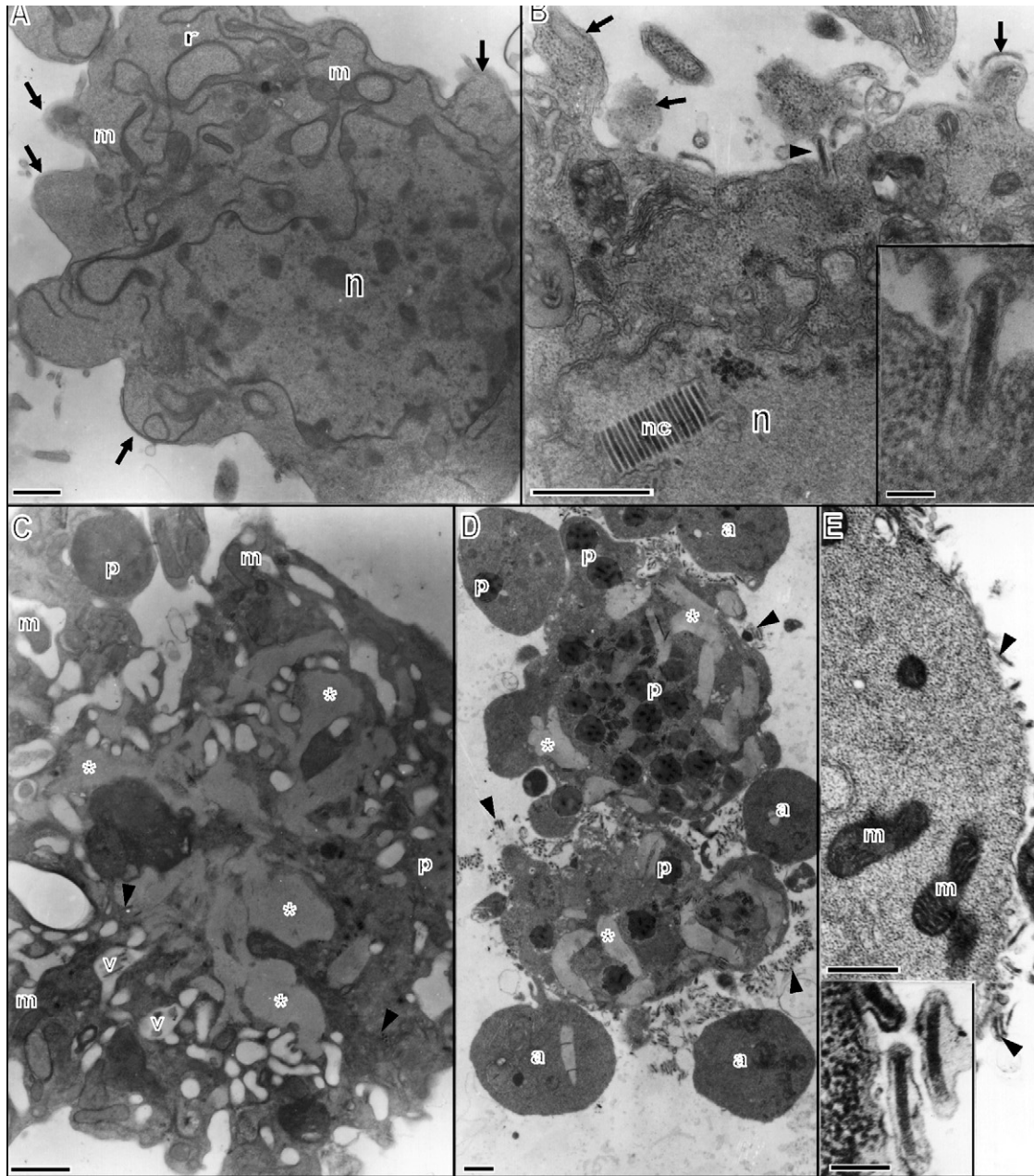


Fig. 5. Transmission electron micrographs of *A. gemmatalis* larval hemocytes in apoptosis induced by vApAg. (A) A putative prohemocyte presenting surface blebbing (arrows). n, nucleus; m, mitochondria and r, rough endoplasmic reticulum. (B) Granular hemocyte type 1. Note the aligned nucleocapsids (nc) in the nucleus (n), surface blebbing (arrows) and a BV (arrowhead) entering the cell by endocytosis (inset). (C) Plasmatocyte in apoptosis. (*), fibrillar aggregates; arrowheads, nucleocapsids; v, vesicles; p, PIBs; m, mitochondria. (D) Unidentified hemocyte in apoptosis. a, apoptotic bodies; (*) fibrillar aggregates; arrowheads, BVs and p, PIBs. (E) Apoptotic body probably derived from a granular hemocyte type 1. Numerous BVs were budding at the plasma membrane (arrowheads) (inset). m, mitochondria. Bars represent 1 μm . Insets bars represent 0.2 μm .

mechanism in insects and its regulation by baculoviruses are important results in this field.

In this work, we have demonstrated that intrahaemocoelic infection of *A. gemmatalis* larvae with vApAg causes apoptosis in hemocytes after 24 h p.i., with the occurrence of progeny production (BVs and PIBs). Despite massive apoptosis at 48 and 72 h p.i., but in correlation to the occurrence of progeny production, BV doses of 10^2 PFU/larva or more induce disease development and death of up to 100% of larvae. However, vApAg LD₅₀ is six-fold higher than the one for AgM-

NPV, which shows correlation between apoptosis occurrence and vApAg infectivity decrease. In comparison to intrahaemocoelic infection with AgMNPV BV, the mean time to death and the LT₅₀ has been extended in an average of 3 days for a same mortality rate (50 or 100% of death), an evidence that apoptosis may retard the advance of the systemic infection.

For orally infected *A. gemmatalis* larvae, it was also demonstrated infectivity reduction for vApAg. The LC₅₀ of vApAg PIBs is five times higher than the LC₅₀ of AgMNPV, the mean

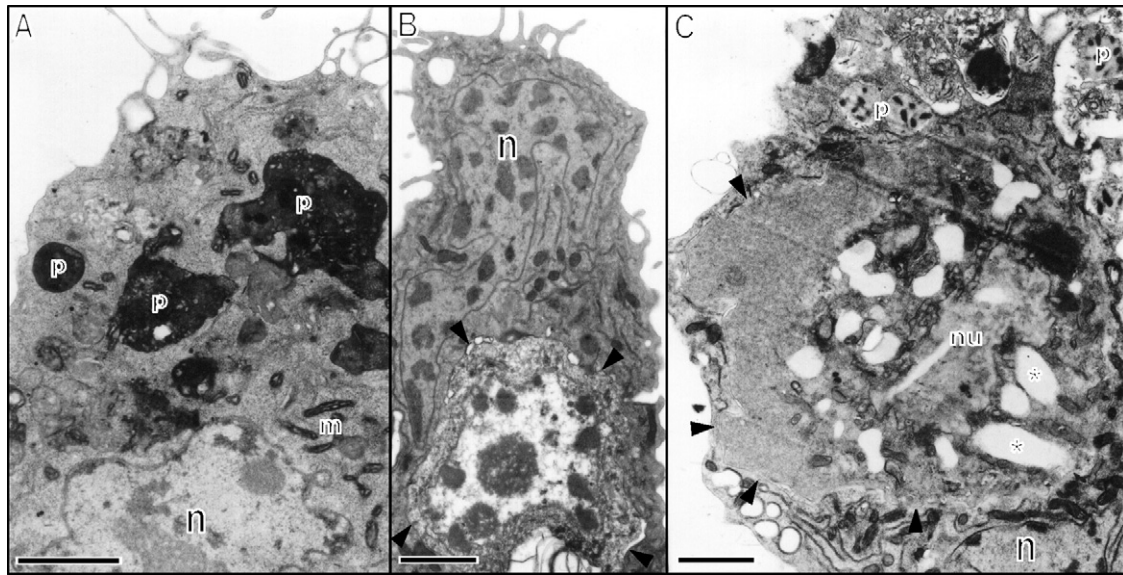


Fig. 6. Phagocytic activity of *A. gemmatalis* larval hemocytes infected with vApAg. (A) Granular hemocyte type 1 whose phagolysosomes contain cell debris (p), n, nucleus and m, mitochondria. (B) A plasmatocyte presenting a phagolysosome containing an entire transversal section of a cell (arrowheads), n, plasmatocyte nucleus. (C) A plasmatocyte that engulfed an infected cell (arrowheads indicate the limits of the phagosome). The same cell also has phagocytosed free PIBs (p), n, plasmatocyte nucleus, nu, engulfed cell nucleus and (*) fibrillar aggregates. Bars represent 2 μm .

time to death was always higher for vApAg in comparison to AgMNPV in a same concentration, as well as the mortality and LT_{50} values. Despite we did not investigate vApAg apoptosis induction by oral infection, the induction of apoptosis by intrahaemocoelic infection and the evident infectivity reduction demonstrated for the two ways of infection permit us to correlate this with a putative apoptosis occurrence in *A. gemmatalis* larvae orally infected by vApAg.

For other systems, apoptosis can reduce more drastically the infectivity in vivo. In *S. frugiperda* larvae, 1000-fold more BV of AcMNPV $p35^-$ and 25-fold more PIBs are required for a LD_{50} in comparison to the wild-type or revertant viruses (Clem and Miller, 1993; Clem et al., 1994). We have shown by electron microscopy that AcMNPV $p35^-$ does not replicate well

in UFL-AG 286 cells or in *A. gemmatalis* hemocytes (Silveira et al., 1999, 2005). In comparison to our data, where similar differences for LD_{50} and LC_{50} between vApAg and AgMNPV were found, we can attribute this to the high susceptibility of *A. gemmatalis* hemocytes to BVs from both wt and mutant virus and the progeny production occurrence despite cell death. For *S. frugiperda*, it has been shown that hemocytes are not a good way for systemic infection spread of AcMNPV, which may be related to the severe infectivity reduction (Clarke and Clem, 2002). However, Haas-Stapleton et al. (2003), using a same source of *S. frugiperda* larvae and AcMNPV strains, have shown a high sensitivity of this insect and its hemocytes to viral infection. The divergence of the data presented by the two groups was attributed to differences in handling techniques.

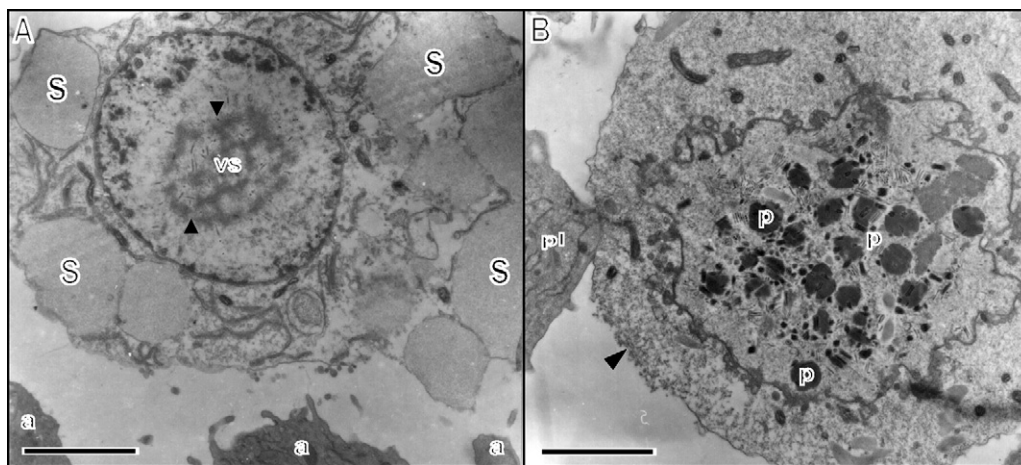


Fig. 7. Necrosis of infected cells. (A) A spherulocyte presenting a mature virogenic stroma (vs) with nucleocapsids assembling (arrowheads). Note the electron density difference between the sph and part of the adjacent cells (a), s, spherules. (B) An oenocytoid presenting PIBs assembling (p). A portion of the plasma membrane was disrupted and part of the cytoplasmic content was extruded (arrowhead). A plasmatocyte (pl) is adhered to the oenocytoid surface. Bars represent 3 μm .

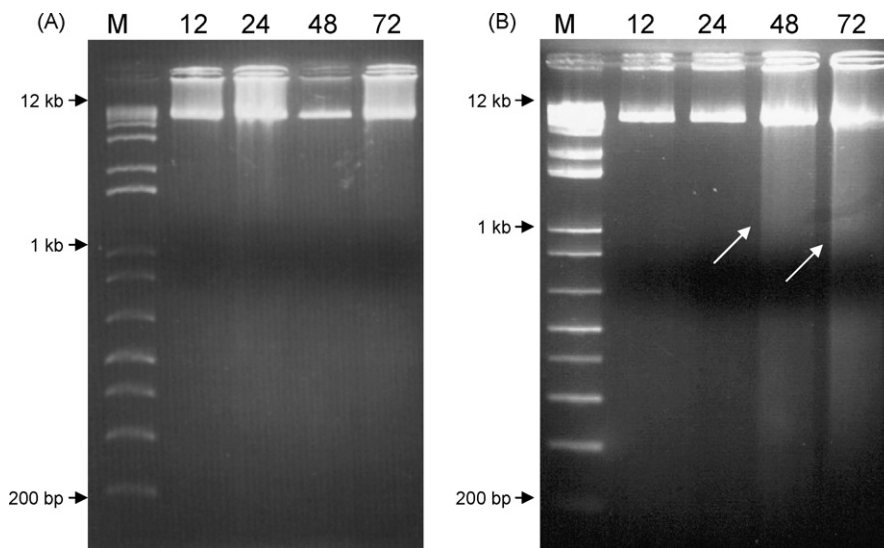


Fig. 8. Three percent agarose gel electrophoresis of DNA extracted from *A. gemmatalis* larval hemocytes infected with AgMNPV (A) and vApAg (B). Each lane contains approximately 2 μ g of DNA. M, 1 kb plus DNA ladder. Arrows indicate smears. Numbers indicate hours post-infection.

The temporal sequence of vApAg morphogenesis in *A. gemmatalis* hemocytes were similar to that observed for UFL-AG-286 cells (Silveira et al., 1999) for AgMNPV in hemocytes (Silveira et al., 2004) and for AgMNPV in other cells (Matos et al., 1999; Pombo et al., 1998). However, symptoms appear earlier through intrahaemocoelic infection than by oral infection, once a high number of BVs is available to infect hemocytes, bypassing natural barriers and defenses such as midgut digestive juices, peritrophic membrane (Granados, 1980) and midgut cell sloughing (Hoover et al., 2000).

Cell fragmentation in apoptotic bodies started at 12 h p.i., but a higher number of apoptotic cells were observed at 48 and 72 h p.i., which indicates a slight delay compared to UFL-AG-286 cells infected with vApAg (Silveira et al., 1999). On agarose gels, there were smears that suggest hemocyte DNA fragmentation at these times, but it was not possible to visualize ladders, as shown for UFL-AG-286 cells (Silveira et al., 1999). Contrary to in vitro conditions, the relative number of apoptotic cells in hemolymph must be lower than in cell culture because of cell turnover and putative differences of susceptibility for the diverse types of hemocytes. Besides this, the intense phagocytosis that occurs in hemolymph may promote a faster secondary degradation of apoptotic bodies DNA into random-size fragments. Together, these factors may have hindered oligonucleosomal fragment resolution and visualization in the gel.

Once cell fragmentation occurred at late times post-infection, the virus cycle of replication was not completely abrogated, allowing BV and PIB production, which correlates with a slighter increase of 6 and 5-fold in vApAg LD₅₀ and LC₅₀, respectively. The vApAg progeny obtained in UFL-AG-286 cells was reduced 100 times when compared to the wild-type virus (Castro and Ribeiro, 2001), showing that apoptosis is correlated with a dropping in progeny production in vitro. Preliminary studies of BVs titration in hemolymph after intrahaemocoelic infection indicate that there is no significant difference between AgMNPV and vApAg levels of progeny pro-

duction in hemolymph for 48 and 72 h p.i., however, additional data is necessary to confirm this tendency. It is possible that in other tissues vApAg does not replicate as well as it does in hemocytes, which may be the determinant of larval death delay.

Besides the usual way of cell fragmentation into apoptotic bodies, at late times post-infection some cells presented nuclear disruption and cell architecture disorganization, associated with an exacerbated expansion of the nuclear envelope. Cytoskeleton changes triggered by baculoviral infection per se (Pombo et al., 1998) associated with apoptosis may be the cause of this unusual event in *A. gemmatalis* hemocytes.

We have found that all hemocyte types of *A. gemmatalis* presented signs of infection, but gh2 appeared to be more resistant to baculovirus infection, as shown previously for the wild-type virus (Silveira et al., 2004). So, there is no difference between AgMNPV and vApAg in relation to the capacity of infection for the different types of hemocytes. Despite infection, PIB assembly and chromatin condensation, sph appear not to be fragmented into apoptotic bodies, a property that may be somewhat related to the unusual morphology of this hemocyte type.

The infection by vApAg generated phagocytic responses by pl and gh1. Both hemocyte types phagocytosed free virions, PIBs, cell remnants, apoptotic bodies and entire cells. Previous observations for these cells infected by AgMNPV revealed pl as the only cell to phagocytose entire cells (Silveira et al., 2004), which was more frequent for vApAg also.

Another event previously reported for intrahaemocoelic infection with AgMNPV and observed for vApAg-infected hemocytes was the necrosis of infected cells, which presented mature virogenic stroma or polyhedra. Together, phagocytosis and necrosis of infected hemocytes are clues for a putative recognition of these virus-infected cells as altered self, and the triggering of cytotoxic responses against them besides apoptosis. Alternatively, necrosis might simply mean that virus is killing the cell. However, for cultivated cells infected with AgMNPV, membrane rupture and necrosis are generally observed only when the

cells are full of PIBs and fibrillar aggregates, and not before the total completion of the viral cycle, as frequently observed in this and in the previous study (Silveira et al., 2004).

Until now, cellular immune responses described against baculoviruses comprises virus uptake in hemolymph, encapsulation and melanization of tracheal infection foci by hemocytes. It has been demonstrated to be important for the circumvention of AcMNPV infection in *Helicoverpa zea* and *Manduca sexta*, as an organism-level strategy of resistance (Trudeau et al., 2001; Washburn et al., 1996, 2000). Besides this, the hemocytes resistance to infection and replication per se is a limiting factor to baculovirus spread in organisms (Trudeau et al., 2001).

For many years, apoptosis induced by mutant baculoviruses in cultivated insect cells had been correlated with infectivity reduction in vivo (Clem, 2001), however only recently this type of cell death was reported in baculovirus-infected insects. AcMNPV was shown to induce apoptosis in diverse tissues of a non-permissive host (*S. litura*) (Zhang et al., 2002). The same happened for a *p35*⁻ AcMNPV recombinant in *S. frugiperda*, which was correlated to infectivity reduction (Clarke and Clem, 2003b). More recently we have described apoptosis induction in *A. gemmatalis* larval hemocytes by a *p35*⁻ AcMNPV recombinant that was also correlated to infectivity reduction (Silveira et al., 2005). These reports confirmed apoptosis as an important anti-viral response in insects and one determinant of host species spectrum (Clarke and Clem, 2003a; Clem, 2001) and so does the present work.

5. Conclusion

This work brought a detailed description of the effects caused by an apoptosis-inducing baculovirus in *A. gemmatalis* hemocytes. It was shown that despite apoptosis induction, vApAg replicates in these cells, with the occurrence of phagocytosis of apoptotic bodies, virions, phagocytosis and necrosis of infected cells. Apoptosis occurrence associated to vApAg infection was correlated with a delay in larval death and infectivity reduction in comparison to AgMNPV-2D wild-type virus, with a difference around five and six times between the LC₅₀ and LD₅₀ for the two viruses. This report reinforces the anti-viral function of apoptosis in insects and demonstrated also the occurrence of other putative anti-viral responses, represented by phagocytosis and necrosis of infected cells.

Acknowledgements

We are grateful to Dr. F. Moscardi (Embrapa Soja) for supplying *Anticarsia gemmatalis* eggs and larvae and Victor H.S. Tibúrcio for assistance in bioassays. This work was supported by CAPES, CNPq, FAP/DF, FINATEC and PRONEX.

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