Structural and ultrastructural studies of Anticarsia gemmatalis midgut cells infected with the baculovirus A. gemmatalis nucleopolyhedrovirus

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Abstract
Anticarsia gemmatalis is a lepidopteran insect susceptible to A. gemmatalis nucleopolyhedrovirus (AgNPV), which is being used in a large scale, in Brazil, as a biological control agent against this serious soybean pest. Baculovirus usually infects its insect host through the midgut epithelium. In the midgut, it replicates in the nuclei of epithelial cells, producing progeny virus and establishing systemic infection. The AgNPV infection of A. gemmatalis midgut was studied using light and electron microscopy. It was observed that AgNPV enters the midgut mainly through columnar cells. Although the virus was not found in the nuclei of columnar cells until late on infection, it is believed that these cells are the primary sites of infection and replication. This fact can be explained by the continuous regeneration of the midgut epithelium. Besides, the infection may be occurring in isolated cells, making it more difficult to be visualized by electron microscopy. At 48 h post infection, hemocytes and tracheoblasts are infected and polyhedra are formed later in these cells, which are the secondary sites of infection.

Keywords: Epithelium; Hemocyte; Lepidoptera; Nucleopolyhedrovirus; Tracheoblast

1. Introduction

Anticarsia gemmatalis is a lepidopteran insect which is a serious pest of soybeans (Moscardi and Correa-Ferreira, 1985 and Moscardi, 1998). As other species from this order, it is highly susceptible to nucleopolyhedroviruses (NPVs). The baculovirus Anticarsia gemmatalis nucleopolyhedrovirus (AgNPV) belongs to the Baculoviridae family of insect virus, with rod-shaped virions containing double-stranded DNA of more than 100,000 base pairs (Arif, 1986). This virus has been used in a large scale to control A. gemmatalis in Brazil. A characteristic of the baculoviruses is the presence of two virus forms during infection, the occluded and budded virus. The occluded form of the virus is produced in the last phase of infection and comprises nucleocapsids embedded in a protein matrix forming an occlusion body (polyhedra) that protects the virus in the environment. Polyhedra are responsible for horizontal transmission of the infection from insect to insect. The budded virus form, which comprises single nucleocapsids with a lipid envelop, is produced before the occluded form, and is responsible for the systemic spread of infection within the insect (Volkman and Keddie, 1990 and Volkman et al., 1995). During the infection process, the midgut epithelium is the first tissue to be

infected. In the midgut lumen, the occlusion bodies are solubilized, releasing the virions by the action of the highly alkaline local cellular environment (Granados, 1980). The released virions attach to microvilli and enter the cell by membrane fusion (Summers, 1971, Kawanishi et al., 1972 and Horton and Burand, 1993). The virus is then transported to the nucleus, where it replicates.

The use of recombinant baculovirus expressing β-galactosidase (LacZ), β-glucoronidase (GUS) and green fluorescent protein (GFP) genes have shown that the midgut columnar cells are the first to appear infected (Engelhard et al., 1994, Flipsen et al., 1995, Knebel-Mörsdorf et al., 1996 and Barret et al., 1998). A recent study has indicated that the insect tracheal system is responsible for the spread of the virus throughout the insect (Engelhard et al., 1994), but the passage of the virus through midgut epithelium is essential for the success of the infection.

This work describes the structural and ultrastructural modifications of the A. gemmatalis midgut epithelium during AgNPV infection.

2. Materials and methods

2.1. Virus and insects

The occluded form of the Anticarsia gemmatalis nucleopolyhedrovirus isolate 2D (AgNPV-2D) (Sieburth and Maruniak, 1988) was used as virus inoculum. A. gemmatalis larvae were provided by National Center of Genetic Resource — CENARGEN (Brasilia, DF, Brazil). They were maintained in plastic cups, at room temperature and fed with an artificial diet (Greene et al., 1976). Third and forth-instar larvae were infected by applying a solution containing the virus (1 × 109 polyhedra) on the surface of the diet.

2.2. Light and transmission electron microscopy

A. gemmatalis larvae were dissected in different intervals of time (0, 1, 2, 3, 6, 12, 24, 48, 72, 96, 120 h) post infection (p.i.). The midguts were removed and sectioned transversely into small pieces, and fixed overnight, in a solution containing 2% glutaraldehyde and 2% paraformaldehyde in 0.1 M sodium cacodylate buffer pH 7.3, at 4°C. The midguts were then washed in the same buffer, and post fixed in 1% osmium tetroxide, 0.8% potassium ferricyanide, 5 mM CaCl2 in 0.1 M sodium cacodylate buffer, at room temperature, for 1 h. Finally the material was dehydrated in acetone and embedded in Spurr's plastic medium (Spurr, 1969). Ultrathin sections were cut on a Reichert Supernova Ultramicrotome. After staining with a saturated solution of uranyl acetate in water and Reynolds's lead citrate (

Reynolds, 1963), the sections were observed using a Jeol 100 C transmission electron microscope at an emission voltage of 80 kV. For light microscopy, thick sections of 1.5 μm were stained with toluidin blue and observed using an Axiophot Zeiss light microscope.

2.3. Scanning electron microscopy

A. gemmatalis larvae infected with AgNPV were dissected in the same intervals of time as described above. Their midguts were opened longitudinally, sectioned, and fixed overnight in a solution containing 2% glutaraldehyde, 2% paraformaldehyde in 0.1 M sodium cacodylate buffer, pH 7.3, at 4°C. Primary fixation was followed by rinsing in the same buffer and fixing for 1 h in the osmium tetroxide-potassium ferricyanide solution. After, the pieces were dehydrated in acetone, dried in a Balzers CPD 030 critical point dryer, coated with gold in a Balzers SCD 050 sputter coater and examined using a Jeol 840A scanning electron microscope, at 10 kV.

3. Results

The midgut epithelium of A. gemmatalis is formed mainly by the columnar and goblet cells (Fig. 1(A)). By scanning electron microscopy two phenotypes of cells were observed: The columnar cells possess many long microvilli whereas goblet cells have mainly smooth surfaces (Fig. 1(B)). A thick peritrophic membrane can be observed between the apical surface of the midgut epithelium and gut contents (Fig. 2(C)). This peritrophic membrane, as observed by scanning electron microscopy, shows an apparent microfibrillar meshwork (Fig. 2(A)).

The apical plasma membrane is modified into cylindrical microvilli displaying dilated tips, from which vesicles apparently pinch off (Fig. 1(C)). Rough endoplasmic reticulum is found in large amounts in the cytoplasm (Fig. 1(D)). Some elements of the Golgi complex occur near the nucleus, which is located at the cell center, whereas free ribosomes predominate in the apical cytoplasm (Fig. 1(D)).

In the goblet cell, the infolded apical membrane displays numerous modified microvilli containing elongated mitochondria (Fig. 1(E)). The cytoplasmic side of these modified microvilli is studded with small particles and many free ribosomes scattered through the cytoplasm. These cells also do not have a well developed endoplasmic reticulum.

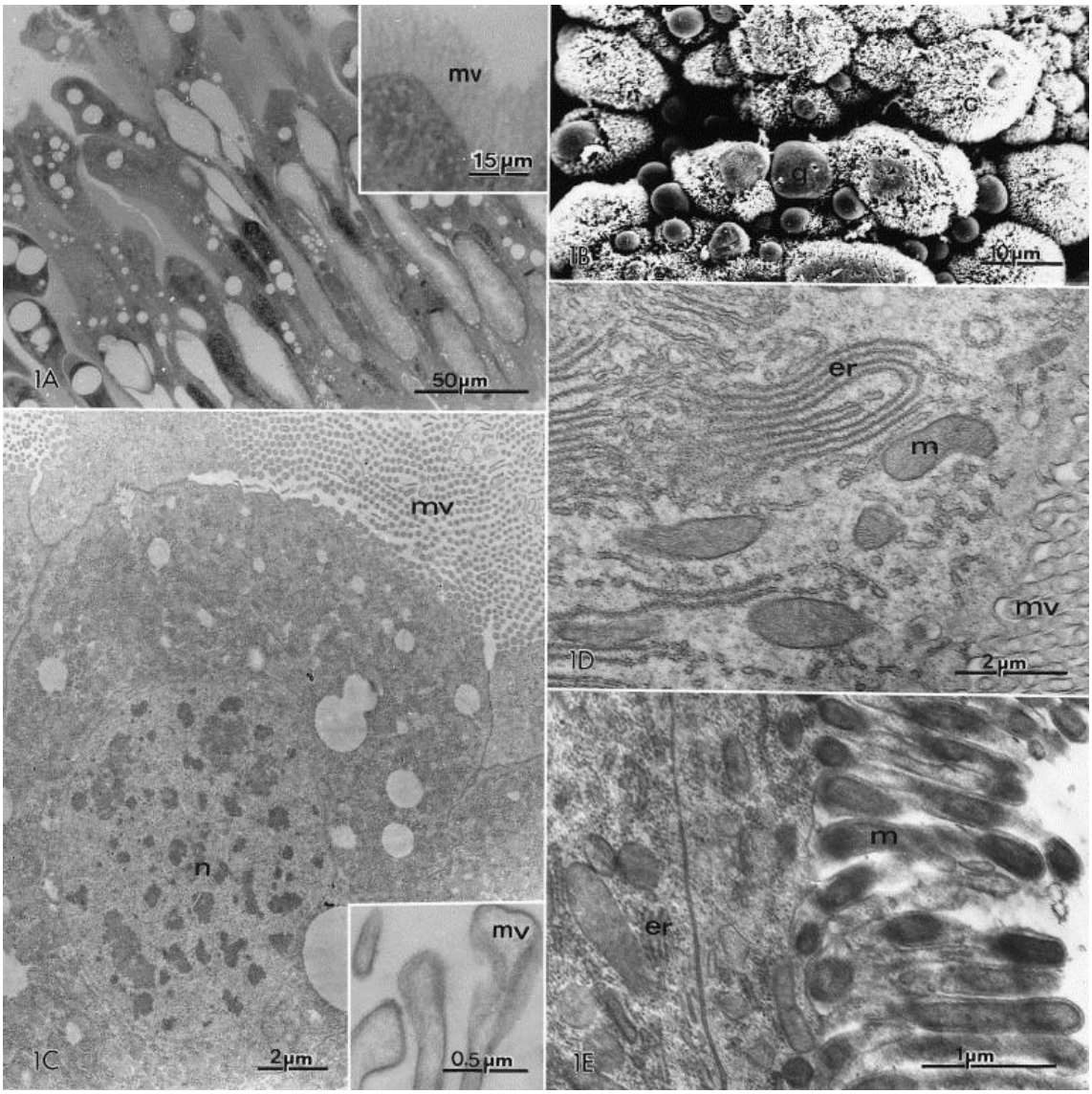

Fig. 1. Midgut of uninfected Anticarsia gemmatalis. (A) Light micrograph of Spurr section showing epithelium with columnar cells. Nucleus (n); microvilli (mv). ×400. Inset: ×1,500; (B) Epithelium with columnar (c) and goblet (g) cells seen with a scanning electron microscope. ×1,400; (C and D) Transmission electron micrographs of columnar cells; (E) goblet cell. Endoplasmic reticulum (er); mitochondria (m); microvilli (mv); nucleus (n). C: ×6,000. Inset: ×29,000; D: ×10,000; E: ×24,000.

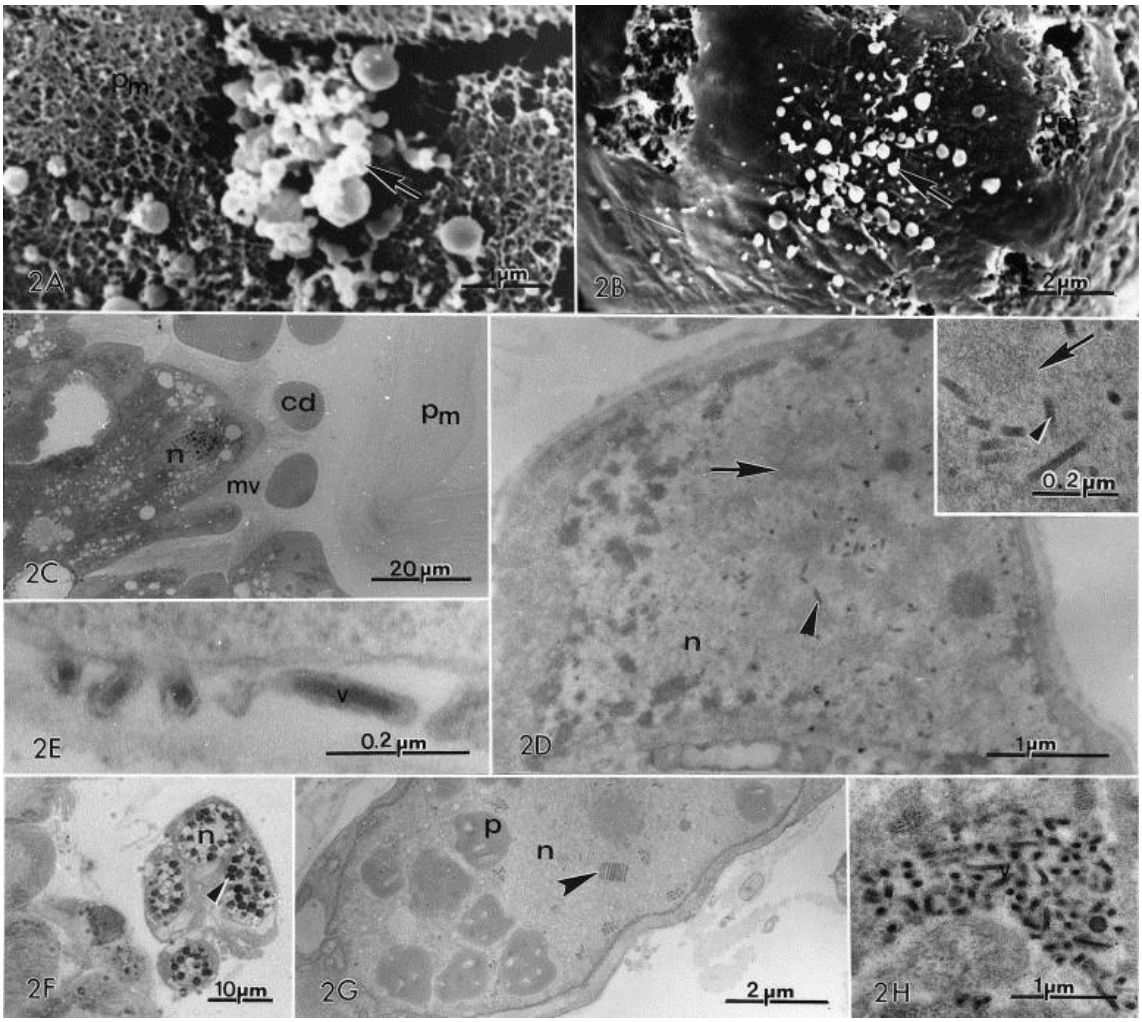

Fig. 2. (A) and (B) Scanning electron micrograph of A. gemmatalis midgut early on infection showing possible polyhedra and/or enveloped nucleocapsids (arrow) adhered to perithophic membrane (pm). ×14,000 and ×6,800; (C) Midgut ephitelium at 48 h p.i. seen with a light microscope. Cellular debris (cd); microvilli (mv); nucleus (n); peritrophic membrane (pm). ×850; (D) Nucleus (n) of tracheoblasts, at 48 h p.i. seen with a transmission electron microscope, showing virogenic stroma (arrow) and nucleocapsids (arrowhead). ×21,000. Inset: ×60,000; (E) Virus particles (v) budding through the plasma membrane of a hemocyte at 72 h p.i. ×125,000; (F) Light micrograph of a hemocyte at 96 h p.i. showing numerous polyhedra (arrowhead) in the nucleus (n). ×1,100; (G) Transmission electron micrograph of a hemocyte with nucleus (n) containing polyhedra (p) and nucleocapsids (arrowhead). ×8,600; (H) Virus particles (v) in the basal membrane of the tracheoblasts. ×18,000.

In the first hours of infection no virus or morphological changes were observed in the cells. However, in the scanning electron microscope at 3 h post infection (p.i.) spherical particles of different sizes were seen adhering to the peritrophic membrane (Fig. 2(A), (B)). These spherical structures may be partially dissolved polyhedra and/or enveloped nucleocapsids, since similar structures were seen when we analyzed dissolved polyhedra in the scanning electron microscope (not shown), whereas the same structures were absent from non-infected control insects. At 48 h p.i. morphological changes were not observed in the midgut epithelium (Fig. 2(C)). However the tracheoblasts and hemocytes appeared infected (Fig. 2(D), (F)). These cells showed hypertrophied nuclei containing the virogenic stroma and

nucleocapsids (Fig. 2(D)). Virus particles were also observed budding through the cytoplasm of these cells at 72 h p.i. (Fig. 2(E)).

Polyhedra were formed at 96 h p.i. in hemocytes and tracheoblasts as it could be observed by light microscopy (Fig. 2(F)). When examined by transmission electron microscopy, the polyhedra showed an electron dense matrix containing enveloped virions that can be formed by one or more nucleocapsids (Fig. 2(G)). Numerous virus particles were also observed in the basal membrane region (Fig. 2(H)).

The midgut epithelium appeared infected only at 120 h p.i. When observed by light microscopy, the tissue showed two morphologically different areas: (1) An infected region in which cells appeared with hypertrophied nucleus, and numerous cells being discarded to the midgut lumen by cell sloughing. (Fig. 3(A)); (2) A non-infected region where the columnar cells appear with structure similar to control cells (Fig. 3(B)). The virus particles and virogenic stroma observed in the nucleus of epithelial cells (Fig. 3(C)) resembled those which are found in tracheoblasts and hemocytes. The hemocytes associated with the basal membrane of epithelial cells appeared infected. Numerous polyhedra were observed in the nuclei of these cells (Fig. 3(B), (D)).

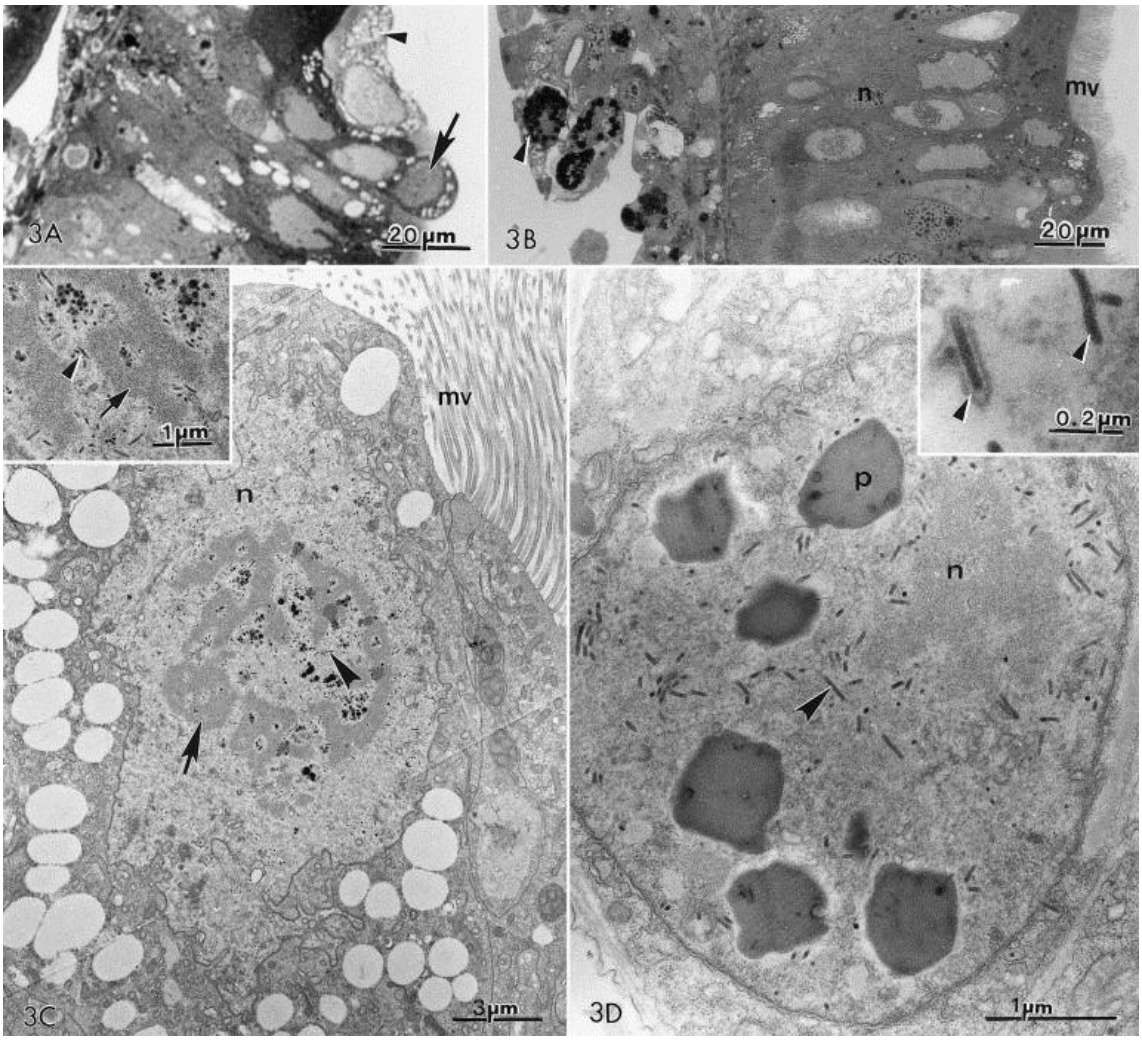

Fig. 3. Midgut cells and hemocytes at 120 h p.i. seen with light microscope (A and B) and transmission electron microscope (C and D). (A) Midgut ephitelium region showing cell with hypertrophied nucleus (arrow) and cells sloughering (arrowhead). ×600; (B) Epithelium with columnar cells. Hemocytes (h) showing polyhedra (arrowhead) in the nucleus; microvilli (mv); nucleus (n). ×700; (C) Columnar cells showing nucleus (n) with virogenic stroma (arrow) and nucleocapsideos (arrowhead). Microvilli (mv). ×5,000. Inset: ×10,000; (D) Polyhedra (p) and nucleocapsideos (arrowhead) are observed in the hyperthophied nucleus (n) of hemocyte. ×22,000. Inset: ×50,000.

4. Discussion

The midgut is the most common virus route of entry into an insect host. The foregut and hindgut are lined with cuticle, thus presenting a barrier to infection. Therefore, only the midgut epithelium appears to play a role in providing the primary sites for virus attachment and entry. However, prior to interacting with midgut epithelial cells, virus must surmount two barriers to infection: the midgut digestive juices and the peritrophic membrane (Granados, 1980).

During the infection of an insect larva by a baculovirus, the ingested polyhedra are solubilized in the alkaline environment of the midgut lumen, releasing occlusion-derived virus

(ODV) (Granados, 1980, Adams et al., 1994 and Volkman, 1997). However, in order to fuse with the plasma membrane these ODVs need to cross the peritrophic membrane, which is a porous network composed of chitin, glycosaminoglycans, glycoproteins, and proteins (Ferreira et al., 1994, Barbehenn and Martin, 1995, Volkman, 1997, Lehane, 1997 and Wang and Granados, 1998). Therefore, peritrophic membrane should constitute a substantial obstacle to midgut infection by baculovirus. The ODV presumably have to penetrate the peritrophic membrane through the pores in order to gain access to the columnar epithelial cells (Barbehenn and Martin, 1995). Previous studies have shown that protein factors found in the occlusion body of baculovirus may be involved in the disruption of the peritrophic membrane (Derksen and Granados, 1988, Greenspan Galo et al., 1991 and Bischoff and Slavicek, 1997).

The primary site of baculovirus infection is the columnar epithelial cells of the midgut (Kawanishi et al., 1972, Keddie et al., 1989, Volkman, 1997 and Flipsen et al., 1995). Our observations on the scanning electron microscope showed that during the first hours after infection, occlusion-derived virus of AgNPV could be observed adhered to A. gemmatalis peritrophic membrane. However, using transmission electron microscopy, no viral particles could be detected in epithelial cells of the midgut early on infection. It was only after 120 h post infection (p.i.) that nucleocapsids could be detected in part of these cells. Several workers have shown the presence of virus particles in columnar cells of the midgut (Kawanishi et al., 1972 and Keddie et al., 1989) early on infection. Other workers have shown, with the help of recombinant baculoviruses containing marker genes, that midgut epithelial cells support viral replication (Volkman, 1997, Flipsen et al., 1995, Washburn et al., 1995, Knebel-Mörsdorf et al., 1996 and Barret et al., 1998) and that the secondary infection was carried out by the insect tracheal system (Engelhard et al., 1994 and Barret et al., 1998). We have shown previously in cell culture that AgNPV expression of very late genes (e.g. polyhedrin) took place between 24 and 36 h p.i. (Pombo et al., 1998). Knebel-Mörsdorf et al. (1996) have shown that expression of a marker gene under the control of an early gene promoter started at 3 h p.i. in midgut columnar cells. The expression of very late genes was observed between 12 and 24 h p.i. and hematocytes were infected only after 20 h p.i. (Flipsen et al., 1995, Knebel-Mörsdorf et al., 1996 and Barret et al., 1998). The failure in detecting viral particles in the midgut columnar cells early on infection is probably due to the relatively small number of cells observed by electron microscopy and that the infection in the midgut is probably not homogeneous, with a relatively small number of foci of infection spread throughout the midgut. Another possibility is that the midgut epithelium is able to clear the infection after the first round of progeny virus production. Washburn et al. (1995) have shown that the infection of midgut columnar cells of Heliothis virescens by a recombinant AcNPV was apparently cleared at 24 h p.i. At this time,

viral infections in H. virescens were restricted to tracheal epidermal cells along with the trachea servicing the midgut. A possibility for clearing of midgut infection was attributed to the rapid replacement of the midgut epithelium by cell sloughing. We have seen the presence of some hypertrophied columnar cell nuclei filled with large number of virus particles and virogenic stroma at 120 h p.i. However no polyhedra were detected. We often observed non-infected cells adjacent to the infected columnar cells, suggesting that the infection process is focused.

Therefore, we believe that the detection of infected midgut columnar cells only at the late stage of infection might be the result of small number of cells infected early on infection, or a loss in the capacity of the midgut to clear the infection by cell shedding and/or to massive secondary infection by budded virus. We detected infected hematocytes and other tissues associated with the basal membrane after 48 h p.i. These results indicate that although the viral particles were not detected in midgut columnar cells early on infection, a few cells might support virus replication and virus particles and/or viral DNA can be transferred to other tissues in order to spread the infection.

In order to confirm that the infection process of the midgut epithelium of A. gemmatalis is focused in a few cells early on infection one would have to construct a recombinant AgNPV harbouring a marker gene in order to follow up the infection in the different tissues of A. gemmatalis in a more detailed way.

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