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The Pathway of Infection of *Autographa californica* Nuclear Polyhedrosis Virus in an Insect Host

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An immunohistochemical study was conducted to detect the temporal infection sequence of *Autographa californica* M nuclear polyhedrosis virus in *Trichoplusia ni* larvae. Staining patterns indicated that the initial infection occurred in the midgut, simultaneously in columnar epithelial and regenerative cells, but that subsequently this tissue recovered. A major envelope glycoprotein stained in a polar fashion when it was expressed in columnar epithelial cells, but not when expressed in other cells types. Systemic infection was mediated by free virus for some tissues whereas infected hemocytes appeared to spread virus to other tissues by an unknown mechanism. A cell to cell spread within several tissues was detected. These results have important implications for baculoviruses engineered for improving their pesticide potential.

NUCLEAR POLYHEDROSIS VIRUSES (Family: Baculoviridae) are double-stranded DNA viruses that cause disease primarily in lepidopteran insects. These viruses are unusual in that at least two structurally different but genetically identical virions (phenotypes) are produced during a successful infection in an insect host. One virion phenotype consists of one or more bacilliform nucleocapsids enclosed in an envelope with numerous virions occluded within a proteinaceous matrix. These occlusion bodies, called polyhedra because of their shape, are formed within the nuclei of infected cells and are visible with a light microscope. They are responsible for the insect to insect transmission of infection. The second virion phenotype consists of a single nucleocapsid in an envelope derived from a viral-modified cellular plasma membrane acquired as virus buds out of a cell (*1*). This phenotype, referred to as extracellular virus (EV), is involved in the systemic infection of a host.

Autographa californica nuclear polyhedrosis virus (AcMNPV) has received much attention because of the use of a promoter of one of its highly expressed late genes in a very efficient expression system (*2*). AcMNPV and other baculoviruses have been less successfully used as biocontrol agents. Their use as pesticides has not fulfilled their promise, in part because of the time required for an infected insect to cease feeding (*3*). The recently acquired ability to manipulate these viruses at the molecular level provides an opportunity to enhance their virulence and their pesticide potential, but the goal of increasing the effectiveness of these viruses as pesticides while maintaining those properties that make them safe, attractive control agents requires a thorough knowledge of all aspects of the virus-host interaction.

We examined the course of infection of AcMNPV in an insect host, *Trichoplusia ni* (cabbage looper), by immunohistochemical staining. Histopathological studies conducted by light and electron microscopy have revealed many aspects of baculovirus infection, replication, and systemic spread in insect hosts (*1, 4, 5*). However, none has used a probe capable of detecting viral antigen expression before polyhedra production in individual cells. This sensitive approach has enabled us to follow the course of infection initiated at biologically relevant doses and examine representative tissue sections from 43 insects by light microscopy.

Insects were infected (*6*) by inoculating them per os with 1 ng of polyhedra derived virus (designated PDV), which caused 95% mortality, and examined from within an hour of exposure to virus until visible symptoms indicated a widely disseminated disease. Developmentally matched uninfected control insects were also examined. Antibodies used for immunostaining were rabbit polyclonal antibody made against PDV (*7*) and several monoclonal antibodies (MAb) reactive with viral structural proteins as determined by immunoblots and immunoprecipitation (*8, 9*). We used the indirect peroxidase-antiperoxidase (PAP) method with diaminobenzidine as the electron donor (*10*). One monoclonal, MAb B12D5, was specific for the 64-kD surface antigen (designated gp64) exclusive to EV; another, MAb 39P10, was specific for a 39-kD major capsid protein present in both phenotypes (*11*). Whole larvae and entire midguts dissected from bled insects were fixed in Bouin's and embedded in Paraplast Plus (Sherwood Medical Industries, St. Louis, Missouri) (*12*). Serial saggital sections were examined for the distribution of viral proteins. In addition, hemolymph (blood) was titered for virus by plaque assay (*13*) from both infected and uninfected insects. Growth and development of the insects were also monitored for all treatments.

In contrast to previous reports (*1, 4*), the results of this study indicated that the initial infection occurred in the midgut in both columnar epithelial and regenerative cells (Fig. 1A). The simultaneous detection of viral proteins in both these cell types implied that they became infected by the parental inoculum. Since the regenerative cells are not exposed to the gut lumen, this observation suggested that this cell type was infected by nucleocapsids or virions that either passed directly through or between cells but were still retained within the epithelium. Several pathways have been proposed by which different baculoviruses may infect their hosts through the midgut (*1*), including rapid passage through or between cells of the epithelial layer directly into the hemocoel (open circulatory system), or replication within columnar epithelial cells before entry into the hemocoel. We saw no evidence of direct passage into the hemocoel on the basis of staining and plaque assay data (Table 1).

Staining patterns in some of the midgut columnar epithelial cells with MAb B12D5 suggested a polar movement of this viral component toward the basolateral area of these cells (Fig. 1B). The distribution of this protein in this region may help to direct the virus to bud from these membranes as has been previously described for other envelope proteins and viruses in cultured epithelial cells (*14*). Previous observations in elec-

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tron microscopic studies (*15*) of baculoviruses in infected midguts have noted the alignment of the nucleocapsids along membranes in the basolateral regions of the columnar cells and subsequent budding in this region. The budded phenotype of the virus must either enter the connective tissue sheath of the midgut or the hemocoel proper for a productive viral infection; thus a directed movement of virus from the columnar cells to these areas is critical. Polarity of gp64 was not observed in any other cell type in this study.

Infected midgut cells were apparently rejected by the insect and sloughed into the gut lumen (Table 1). As columnar cells are more proximal to the lumen than regenerative cells, infection of the latter with their longer time in place may be critical to successful systemic invasion. We observed groups of infected regenerative cells behind an infected columnar epithelial cell but in front of other apparently uninfected regenerative cells (Fig. 2A). We interpreted this as a proliferative response by the gut to rid itself of injured (infected) cells. Indeed, the gut epithelial tissue appeared to be nearly fully recovered by 61 hours after infection, as judged by the reduction of the number of stained midgut cells (Fig. 2B) and the continued normal growth of infected insects. For this recovery strategy to be effective, the sloughing of infected cells and replacement by uninfected cells must outpace virus replication and release. Apparently this was the case. Another phenomenon that may have aided in the recovery of the midgut was a burst of cell division of midgut epithelium and concomitant cell sloughing that accompanied the molt to fifth instar larvae (Fig. 2C).

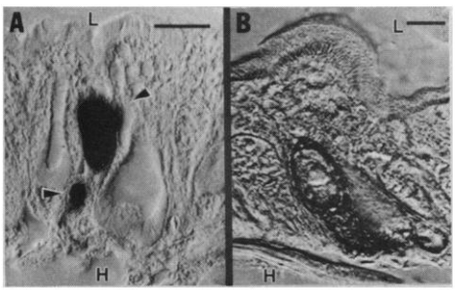

Fig. 1. Immunohistochemical staining of 5-μm tissue sections prepared from nuclear polyhedrosis virus-infected *T. ni*. (**A**) Earliest staining (16 hours after infection) was seen in columnar and regenerative midgut cells. Staining was restricted to the nuclei (arrows) when a MAb reactive to a major capsid protein was used (n = 13). (**B**) A polar distribution of envelope glycoprotein (gp64) in a midgut columnar epithelial cell detected by MAb reactive with this protein. Polarity was not observed in any other cell type (n = 24). L, gut lumen; H, hemocoel. Bar, 10 μm.

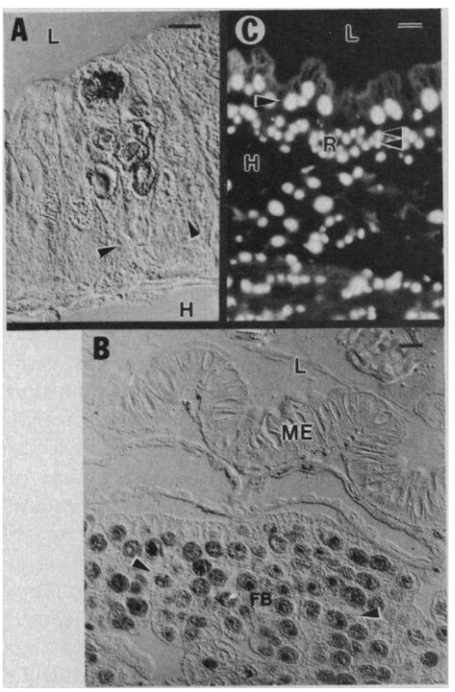

Fig. 2 Changes observed in the midgut during the course of infection. (**A**) An infected columnar epithelial cell and associated regenerative cells displaced toward the gut lumen with replacement by apparently uninfected cells (arrows) (36 hours after infection) (n = 7). (**B**) Uninfected (recovered) midgut epithelium (ME) with widespread hemocoelic infection (arrows) (90 hours after infection) (n = 9). (**C**) Nuclear DNA staining with 4′,6 diamidino-2-phenylindole (DAPI) during the molt from fourth to fifth instar demonstrating a multiple layer epithelium consisting of regenerative cells (double arrows) and differentiated cells (single arrow) in both infected and uninfected insects (n = 6). L, gut lumen; N, nucleus; H, hemocoel; FB, fat body; R, regenerative cells. Bar, 30 μm.

The detection of viral protein in the midgut paralleled the detection of infectious virus circulating in the hemocoel (Table 1). The cells in which viral protein was next detected were those that are part of the midgut connective tissue sheath. Although not completely characterized, this sheath contains muscle cells, tracheoblasts, and fibroblast-like cells (*16*). With a light microscope we could not determine whether any of these were preferentially infected during this early course of the disease, although at later times all of the cells contained in the sheath appeared infected.

Once EV circulated with the hemolymph in the hemocoel, susceptible tissues and organs presumably were exposed to virus almost simultaneously. In spite of this, the temporal staining pattern suggested that some cell types were more readily infected than others (Table 1). We consistently observed staining within the hemocoel in the hemocytes (blood cells) and tracheal epithelium 31 to 40 hours after infection. Some of

Table 1. Percentage of insects with infectious virus in the hemolymph determined by plaque assay and detection of viral antigens in tissues by immunostaining. Inf, infected; +, staining detected; −, no staining detected; n, number of insects tested; nd, not done. Time is hours after infection.

Time	Plaque assay		Tissue assay		Detection of viral antigens in tissues						
					Midgut						
	Inf (%)	n	Inf (%)	n	Columnar cells	Regenerative cells	Connective layer	Hemocytes	Trachea	Epidermis	Fat body
0–12	0	8	0	7	−	−	−	−	−	−	−
13–17	38	8	30	10	+	+	−	−	−	−	−
18–30	94	16	90	10	+	+	+	−	−	−	−
31–40	100	8	100	3	+	+	+	+	−	−	−
41–60	nd	nd	100	4	+	+	+	+	+	+	+
61–100	nd	nd	100	9	−/+*	−/+*	+	+	+	+	+

*−/+, 4/9 insects had a few infected columnar and regenerative cells remaining.

the infected hemocytes appeared to be adhering to organ surfaces, especially the epidermis (Fig. 3A). Infection of epidermis was detectable 6 to 12 hours later as widely scattered foci of one or two cells (Fig. 3B). Subsequently the infection within the epidermis seemed to radiate outward from these foci, producing a patchwork pattern of infected and uninfected tissue (Fig. 3C). Staining was rarely observed in the fat body before 45 to 50 hours after infection although it is one of the most abundant tissues at this stage of larval development. The pattern seen in this tissue indicated that the initial infection occurred adjacent to the infected epithelium of trachea that penetrated into this tissue (Fig. 4). Staining also was detected in other tissues, but the temporal and distributional patterns were less consistent among the specimens. Of the remaining tissues, staining was frequently observed in the sarcolemma of muscle, the testicular sheath, less frequently in the connective tissue sheath of ganglia, and rarely in either neurons or glial cells of the brain. In most of these tissues, associated tracheal cells appeared to be the predominantly infected cells. Staining was also rarely encountered in the Malpighian tubules or silk glands. The involvement of these tissues has been reported previously (*5*).

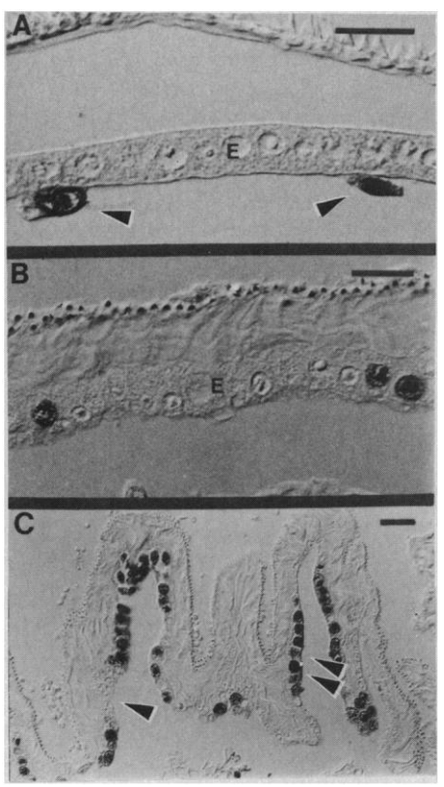

Fig. 3. Observations on the temporal pattern of pathology in the epidermis during the disease. (**A**) Infected hemocytes (arrows) adhering to epidermis (E). (**B**) Dispersed one or two cell foci of infected epidermal cells. (**C**) Expanded foci of infected epidermal cells (double arrows) interspersed with uninfected regions (single arrows) (n = 9). Bar, 20 μm.

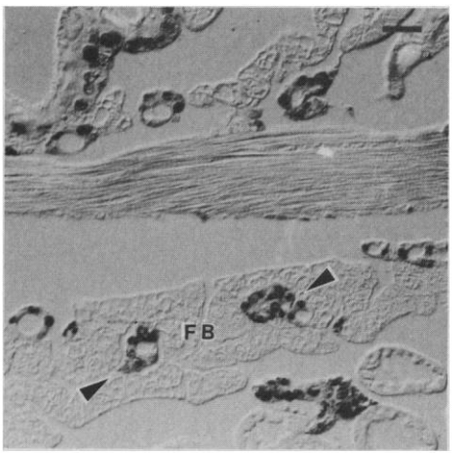

Fig. 4. Infected trachea (arrows) intimately associated with uninfected fat body (FB). Cell to cell spread by virus is necessary where the tracheal cells are precluded from contact with the hemocoel and circulating virus by other tissue (n = 3). Bar, 30 μm.

The sequential nature of the temporal staining pattern seen in the hemocoel in many of the tissues may be a consequence of the existence of physical barriers to virus infection. Basal lamina of various thicknesses surround virtually all of the tissues with the exception of hemocytes in insects (*17*). This secreted layer has been shown to have selective permeability (*18*) and the capacity to inhibit baculovirus entry as demonstrated by increased viral penetration after collagenase digestion reduced the integrity of this layer (*19*). Apparently adherence of infected hemocytes to the basal lamina of various organs augmented virus penetration. Adhering hemocytes have been described as contributing to the formation of this layer in uninfected insects (*20*). After initial infection the virus appeared to spread from cell to cell as judged by the expanding foci of stained cells seen in the epidermis. In previous cell culture studies EV has been shown to enter by adsorptive endocytosis (*21*), but these cells have membranes exposed to the culture medium. In tissues the membranes of adjacent cells are in apposition or are bounded by basal lamina when in contact with another tissue. How virus is transferred from cell to cell in these instances is unknown.

In summary, the initial infection of *T. ni* by AcMNPV appeared to occur in both the columnar epithelial and regenerative cells of the midgut. Budded virus from these cells appeared to both infect cells in the midgut connective tissue sheath and enter the hemocoel. Within the hemocoel, hemocytes and the tracheal epithelium were infected first, with subsequent spread to most other tissues. Many of these tissues appeared to be infected as the result of contact with infected hemocytes or tracheal cells. Infection within the hemocoel therefore was mediated both by circulating EV and infected cells.

Our results suggest two possible targets for viral modification for improvement of pesticide potential: the midgut and the basal lamina. If enough damage could be inflicted on the midgut by virus infection such that the midgut's repair capabilities were overcome, or if the basal lamina could be partially disrupted by expressed foreign gene products to aid the spread of infection of EV, insect feeding could be arrested much more rapidly. AcMNPV thus modified might then be an effective, environmentally safe biocontrol agent.

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