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The insect tracheal system: A conduit for the systemic spread of *Autographa californica* M nuclear polyhedrosis virus

(baculovirus movement/*Trichoplusia ni*/tracheae/viral pathogenesis)

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ABSTRACT **Baculoviruses establish systemic infections within susceptible insect hosts, even though host tissues are surrounded by basal laminae, extracellular matrices that exclude particles smaller than these viruses. Using a recombinant *Autographa californica* M nuclear polyhedrosis virus containing a *lacZ* reporter gene under the control of a constitutive promoter, we followed the progression of infection in *Trichoplusia ni* larvae. We discovered that infection of the larval insect tracheal system (and not hemocytes, as thought previously) provides the major conduit for this virus to pass through basal laminae and to spread throughout the host. Tracheal epidermal cells, the only known cellular components of the tracheal system, share a common lymph system. Locally these cells contact one another by interdigitating cytoplasmic extensions called epidermal feet. These two features of the tracheal system are likely to facilitate the rapid systemic spread of the virus. The findings reported here have major implications for the fields of insect pathology and biological control and usher in an important consideration regarding host-range factors.**

Nuclear polyhedrosis viruses (NPV) (family Baculoviridae) are enveloped double-stranded DNA viruses that infect only arthropod hosts, primarily lepidopteran insect larvae. NPVs are unusual among viruses because they produce two phenotypes, one that transmits infection between hosts and one that spreads infection within the host. The first phenotype consists of one or more enveloped virions, each containing one or more nucleocapsids sequestered within a crystalline matrix of protein. Such viral occlusions are called polyhedra. As with the spore stages of many bacteria and fungi, polyhedra resist desiccation and allow for temporal escape from inhospitable environments. After ingestion by susceptible larval insect hosts, polyhedra rapidly dissociate in the alkaline gut juices and release occlusion-derived virus (ODV). Even though ODV lack surface spikes or peplomers, they enter midgut columnar epithelial cells by membrane fusion (1–3). These infected epithelial cells produce the second phenotype, budded virus (BV), which buds through the basal cell membrane at sites containing gp64, a viral-encoded glycoprotein that forms spikes on the surface of BV. These gp64-containing structures mediate entry of BV into target cells by promoting fusion of the BV envelope and endocytic vesicular membranes (4–6).

Early investigations of baculovirus infections focused on host pathology and the potential use of baculoviruses for biological control purposes. Accurate descriptions of the progression of pathogenesis in virus-infected insects, however, were hampered by the limits of the light and electron microscopy techniques commonly used, both with and without accompanying immunohistochemistry. Rare events are difficult to capture by using these techniques because they require meticulous serial sectioning and the three-dimensional reconstruction of hundreds, if not thousands, of sections for each insect. Strategies commonly used to reduce the enormity of this task have included infecting with very large doses of virus, sampling only a small fraction of the insect tissues, and limiting the number of insects examined. All of these alternatives have major pitfalls that may bias the accuracy of the information they impart or limit the conclusions that can be derived from the data.

One extremely important but poorly understood event concerns how baculoviruses penetrate the basal lamina, a fibrous extracellular matrix surrounding all insect tissues (7), to achieve systemic spread within infected hosts. The basal lamina is negatively charged and impenetrable to particles >15 nm (8). This structural feature should exclude the free passage of virions that measure 30–35 nm × 250–300 nm, and indeed several authors have observed baculovirus virions apparently trapped in the space between the basal membrane of the midgut epithelium and its associated basal lamina (9, 10).

To elucidate the mechanism of basal lamina penetration and to follow the progression of infection within an insect in general, we developed a recombinant *Autographa californica* NPV containing a reporter gene cassette (designated AcMNPV-hsp70/lacZ), which permits visualization of the viral-infection pathway in larval hosts challenged with biologically realistic dosages. Using AcMNPV-hsp70/lacZ, we could quickly and thoroughly detect infected tissues from individual primary target cells to widespread systemic infection in larval whole-mount preparations. Although tracheal cells have long been known to be a secondary target of infection (11), we discovered that systemic infection throughout the host insect is mediated by a specific tracheal system tropism.

MATERIALS AND METHODS

Construction and Characterization of AcMNPV-hsp70/lacZ Recombinant Virus. AcMNPV-hsp70/lacZ was constructed by cotransfecting Sf9 cells (*Spondoptera frugiperda* clone 9) in culture (12) with the transfer vector designated pAcLV1 and wild type (E2 strain; ref. 13) viral DNA (Fig. 1). The recombinant virus thereby contained all the wild-type genes of AcMNPV, including polyhedrin, plus an additional insertion of β-galactosidase under the control of the *Drosophila hsp70* promoter. This promoter is constitutive in larval *Trichoplusia ni*. AcMNPV-hsp70/lacZ was determined to be biologically similar to the parent strain E2 with regard to infectivity and

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Abbreviations: AcMNPV, *Autographa californica* M nuclear polyhedrosis virus; ODV, occlusion-derived virus; BV, budded virus; hpi, hours postinoculation.

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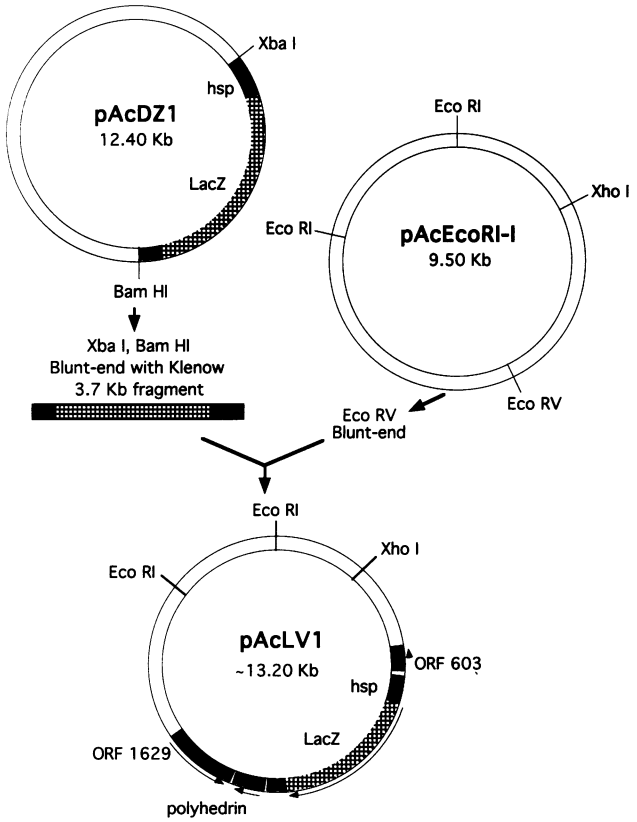

FIG. 1. Construction of a *Drosophila* hsp70–β-gal reporter gene in a transfer vector. The hsp70–β-gal chimeric gene was excised from the plasmid pAcDZ1 (14) and ligated at the *Eco*RV site upstream of the polyhedrin gene in the *Eco*RI I fragment of the AcMNPV genome (15). The resulting transfer vector was designated pAcLV1. ORF, open reading frame.

pathogenesis in both cell culture and in *T. ni* larvae (16, 17). BV titer was determined by immunoplaque assay using IPLB-Sf-21 cells (18).

Inoculation of *T. ni* Larvae. *T. ni* larvae (Lepidoptera: Noctuidae) were reared as described (17) and were infected immediately after the molt to the fourth instar. All insects used were carefully matched developmentally. Insects molting from third to fourth instar were observed slipping out of their cuticles and inoculated with either ODV or BV within 10 minutes of this event. ODV [0.036 pg (LD_{74}) or 1.0 pg (LD_{95}) per insect] was administered orally with a microapplicator that delivered the inoculum directly into the anterior midgut (17). BV [0.005 plaque-forming unit (LD_{50})] was administered directly into the hemocoel through the planta of the first right abdominal proleg (17). Larval cohorts of 18–25 insects were sacrificed and processed at various times from 3 to 70 hr postinoculation (hpi) and examined for the appearance of blue cells expressing lacZ; 308 ODV-inoculated and 110 BV-inoculated larvae were examined in total.

Preparation and Examination of Larvae for LacZ Expression. Larvae were prepared for observation by securing them individually onto wax plates with minutin pins through the head capsule and terminal abdominal segments. Specimens were covered with cytoskeleton extraction buffer (CEB: 10 mM Pipes/60 mM sucrose/100 mM KCl/5 mM $Mg(OAc)_2$/1 mM EGTA/distilled water, pH 6.8), and a single longitudinal incision was made through the dorsal cuticle along the length of the body. For insects inoculated with ODV and sacrificed 3 to 24 hpi, the entire midgut was excised intact and immediately placed in fixative (2% paraformaldehyde/CEB) at 22°C. Samples of hemolymph were taken from insects inoculated with BV at 12, 16, 20, and 24 hpi. Hemocytes were attached to multiwell tissue culture plates for 1 hr before fixation for 1 hr at 22°C. Whole-mounts were prepared for larvae infected with ODV for >24 hr and all larvae inoculated with BV. Whole-mounts were created by making small lateral incisions at the ends of a dorsal longitudinal incision, pinning back the cuticle, and exposing the internal tissues. Immediately after, CEB was removed, and the preparations were infused with fixative for 12–24 hr. All tissue samples were then rinsed in CEB for 1 hr and infused with enhancement buffer [5 mM $K_4Fe(CN)_6$/5 mM $K_3Fe(CN)_6$/2 mM $MgCl_2$ in distilled water] containing 5-bromo-4-chloro-3-indolyl β-D-galactoside at 1.5 mg/ml for 6–12 hr in total darkness. Samples were observed with standard dissection (×10–40) and compound (×100–480) microscopes.

RESULTS AND DISCUSSION

Blue cells were first observed at 4 hpi in the midgut epithelia of insects inoculated orally with ODV (two replicates; n_1 = 18, n_2 = 25). At this time point, 11% and 12% of inoculated insects contained blue cells, all of which were single differentiating or mature columnar cells (Fig. 2*A*) (20). By 12 hpi ≈80% of inoculated insects (n = 25) were expressing lacZ. Multicell foci were first observed at 12 hpi and by 16 hpi, 13 of 16 insects expressing lacZ (81%) contained multicell foci. Presumably these multicell foci were generated from the infection of neighboring cells by progeny virions from primary target cells, although infection by parental nucleocapsids cannot be ruled out entirely because the occluded form of AcMNPV is a multinucleocapsid virus. The multicell foci seen between 12 and 16 hpi consisted primarily of mature columnar, differentiating, and regenerative epithelial cells, but infection of the first non-midgut epithelial host cells, tracheoblasts, was also detected (16% and 54% of inoculated insects at 12 and 16 hpi, respectively). Extensive local infections of tracheoblasts and tracheal epidermal cells associated with midgut epithelial foci were visible by 24 hpi in 83% (n = 24) and 64% (n = 25) of inoculated insects (Fig. 2*B*). By 36 hpi, we observed infection in hemocytes and dispersed tracheoblasts in 54% and 83%, respectively, of insects examined (n = 24). By 48 hpi, widespread infection of the tracheal system (Fig. 2 *C* and *D*) and hemocytes was evident in 75% of the insects examined (n = 24); infected fat body was detected in 50% of these same insects (Fig. 2*E*). We noted that infections of the tracheal system always originated from tracheoblast cells and proceeded linearly along tracheal branches (Fig. 2*D*). Curiously, at 48 hpi, infection of other tissues was observed only rarely, and the midgut epithelia of 77% of infected larvae had been cleared. By 70 hpi, however, all infected insects (n = 18) showed some degree of infection in muscle fibers, salivary glands, malpighian tubules, gonads, cuticular epidermi, and midgut epithelia. When infection was initiated by injection of BV into the hemocoel, infected hemocytes and tracheoblasts were detected simultaneously as early as 20 hpi in 40% of the insects examined (n = 20), indicating that populations of tracheoblasts and hemocytes were similarly susceptible to infection (Fig. 2*F*).

Early infection of and rapid dissemination through the tracheal system provide evidence that the insect respiratory system plays a pivotal role in the systemic spread of this virus. The tracheal system facilitates the exchange of gases between all tissues and the external environment through invaginations of the outer body wall that form gas-filled tubes, called tracheae. The tracheae are lined with a chitinous cuticle secreted by a supporting epidermal layer, much like the external insect integument. Tracheae bifurcate upon entering the insect, creating increasingly finer tubes, until the lumen of each tube becomes an intracellular structure called a tracheole. The cells harboring tracheoles are newly produced daughter cells of the insect tracheal epidermis, called

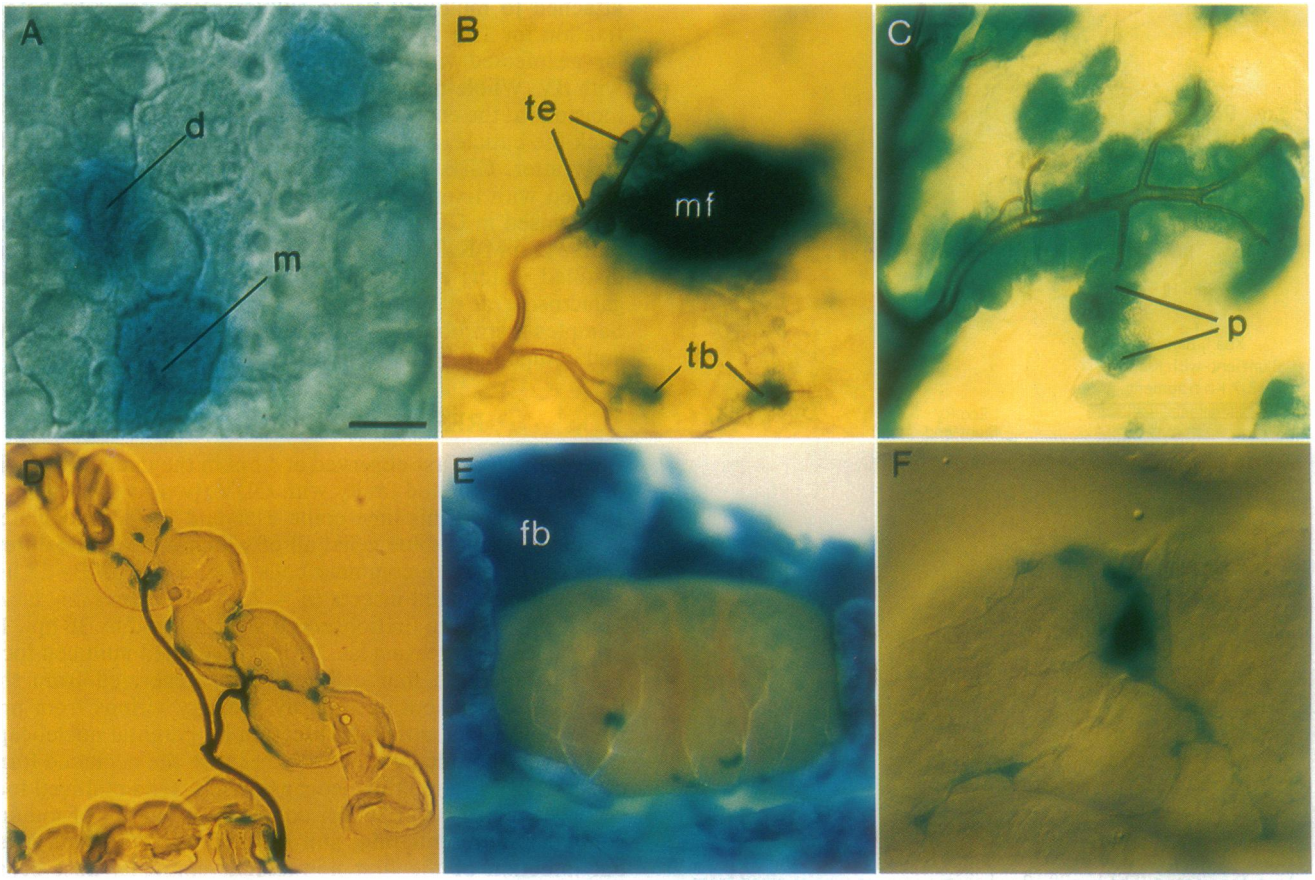

FIG. 2. Detection of lacZ expression in whole-mounts of AcMNPV–hsp70/lacZ-infected *T. ni*. (*A–E*) Larvae inoculated orally with ODV. (*A*) Apical view of a midgut epithelium 6 hpi. Early infection is limited to discrete mature (m) and differentiating (d) columnar cells. The basal lamina has been removed to facilitate microscopy (20). (Bar = 14 μm). (*B*) Basal view of intact midgut at 24 hpi. Infection has spread from a multicell focus (mf) within the midgut epithelium to associated tracheal epidermal cells (te) and to distant tracheoblasts (tb). (Bar = 32 μm.) (*C–E*) Tissue at 48 hpi. (*C*) Infection has spread rapidly within the tracheal epidermis where many cells contain polyhedra (p). The midgut epithelium is no longer infected. (Bar = 16 μm). (*D*) Infected tracheoblasts on the surface of a malpighian tubule. (Bar = 220 μm.) (*E*) A testis is surrounded by heavily infected fat body (fb). The only infected cells associated with the testis are tracheoblasts. (Bar = 64 μm.) (*F*) An infected tracheoblast from an insect injected intrahemocoelically with BV, 24 hpi. At this time only hemocytes and tracheoblasts were observed to express lacZ in insects infected by this route. (Bar = 16 μm.)

tracheoblasts (21). Tracheoblasts have many fine processes (Fig. 2*F*), each of which may contain a tracheole; one process is always linked to the larger tracheal lumen, whereas the other processes contact cells of other tissues. As an insect grows, the tracheal system maintains contact with new cells by mitotic division of tracheoblasts that are capable of taxis toward cells experiencing respiratory distress (22). Maina (23) demonstrated that the tracheoblasts of the grasshopper, *Chrotogonus senegalensis*, penetrate the basal lamina surrounding the midgut and establish direct membrane contacts with midgut epithelial cells. Our work clearly indicates that tracheoblasts are infected as immediate secondary host cells and suggests that the virus circumvents the basal lamina barrier by intracellular passage through these cells. Furthermore, free virions in the hemocoel do not appear to enter other tissues directly but rather appear to depend on passage through tracheoblasts as well; thus systemic infections appear to be mediated by tracheoblasts that serve as portals for the bidirectional passage of BV across the basal lamina barriers.

The rapidity of transmission of infection within the tracheal system and the fat body between 24 and 48 hpi is difficult to explain by a direct cell-to-cell infection mechanism. Locke (24) observed two epidermal structures in larvae of *Calpodes ethlius*, another lepidopteran insect, that could explain rapid viral spread through the tracheal system (Fig. 3). (*i*) Interdigitating lateral epidermal cell membranes form a network of interconnecting channels containing lymph. These lymph channels are bordered apically by septate membrane junctions and basally by negatively charged borders and basal lamina. (*ii*) Epidermal cells form basal processes called epidermal feet; these processes invaginate the basal membranes of surrounding epidermal cells and connect cells several cell bodies apart. Locke (24) suggested that both the lymph system and the epidermal feet could facilitate tissue-specific cell communication over long distances. These structures also could facilitate the rapid spread of baculovirus infections. Locke also observed interconnected lateral lymph spaces within the fat body of *C. ethlius* (25), and significantly, these lymph spaces were intimately associated with the tracheal system.

AcMNPV infection of tracheoblasts offers three advantages to the virus. (*i*) Tracheoblasts provide a permanent infection foothold because these cells are not sloughed, as are cells of the midgut epithelium. Keddie *et al.* (26) observed, as did we, that the midgut epithelium of *T. ni* challenged with AcMNPV recovered from the initial infection. The mechanism of recovery is thought to be cell sloughing, either at a basal rate or at an accelerated rate in response to infection. (*ii*) Tracheoblasts are in direct contact with the tracheal epidermis, which not only supports infection (Fig. 2*C*) (11) but also is intimately associated with all other tissues. Thus, the tracheal system can function both as a productive target tissue and as a conduit to other tissues. (*iii*) Finally, infection of tracheoblasts allows virions to circumvent the basal lamina barriers.

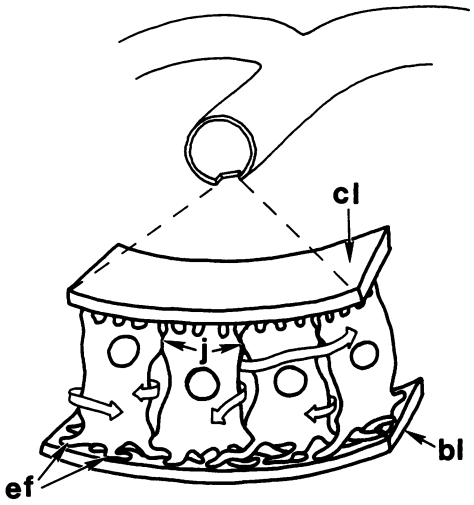

FIG. 3. A section of a trachea illustrating the relationship between the cuticular lining (cl), tracheal cells and their epidermal feet (ef), basal lamina (bl), and the tracheae-specific lymph system that may facilitate the rapid movement of infection. Arrows indicate movement of lymph within a common extracellular space. The lymph space is bordered apically by septate junctions (j) and basally by the basal lamina. Epidermal feet provide direct membrane contact between cells several cell bodies apart. This figure is a modification of a figure in ref. 24.

Previously it was thought that infected hemocytes were the principal vehicles for the spread of infection within insects (19, 26). Our results suggest another model for AcMNPV systemic infection. Midgut epidermal cells are primary target cells, but the underlying basal lamina prevents the progeny virions they produce from directly entering the insect hemocoel. Instead, passage through the basal lamina is facilitated by immediate secondary infection of tracheoblasts. These cells cross the basal lamina and can deliver virus both to the tracheal epidermal cells and to the hemocoel of the insect. The very rapid spread of infection through the tracheal system probably is facilitated by the epidermal lymph system and epidermal feet. Hemocytes are infected and amplify virus progeny in the open hemocoel, but the basal laminae apparently prevent virions from directly entering tissues, except for tracheoblasts. These results unequivocally demonstrate that the tracheal system is the most important conduit for AcMNPV spread in *T. ni*, and additional studies have revealed the same mechanism for systemic infection of another highly susceptible host, *Heliothis virescens* (J.O.W., unpublished work). The rapid movement of AcMNPV out of the midgut and the subsequent use of the insect respiratory system are elegant evolutionary adaptations for maximizing production of viral progeny. Initial clearing of virus from the midgut preserves this tissue during concurrent viral infection of less vital tissues, such as the fat body. Infected larvae continue to feed and to grow until very late in infection and thereby provide additional host biomass that ultimately can be exploited for viral amplification.

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