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Segmental pairs of giant insect cells discharge presumptive immune proteins at each larval molt

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Abstract

A pair of massive secretory cells exists within each thoracic and the nine abdominal segments of *Manduca* larvae. Each of these cells is nestled between the dorsal integument and underlying muscles. Contents of large vacuoles in these cells are abruptly discharged at each molt and have always been considered to contribute to shedding and/or formation of cuticle. Peanut agglutinin is a specific lectin label for these secretory vacuoles; vacuoles label intensely immediately before each molt as vacuoles

attain their maximal size. Contents of vacuoles are restored after each molt and throughout most of each intermolt. During the molt cycle these cells secrete contents of their vacuoles into the interior hemocoel rather than onto the exterior cuticle. Vacuoles discharge via a distinctive mechanism involving partitioning of contents into numerous vesicles that move to the cell surface. Dermal secretory cells were dissected from larvae before and after the 4^{th} to 5^{th} instar molt. Proteins from pre-molt and post-molt secretory cells were separated by two-dimensional electrophoresis to establish which proteins are discharged at the molt. While secreted proteins are novel, all have presumptive roles in immune responses. Dermal secretory cells may represent a new, unsuspected component of the innate immune system that release their proteins during the vulnerable molting period of an insect's life.

Keywords: dermal gland; secretory cell; molt; glycosylated proteins; innate immunity

Introduction

Despite their imposing sizes, dermal secretory cells of larval dermal glands have remained cells with uncertain functions. Each dermal gland consists of three cells - duct cell, saccule cell, and secretory cell. Secretory cells are present throughout larval life and grow in size without any cell divisions. The size of each secretory cell and its vacuoles are closely coupled to each molt cycle and hormonal titers (Horwath and Riddiford, 1988; Lane et al., 1986); at each molt cells attain their maximum size for the preceding instar. At each molt, the contents are abruptly expelled, and secretory cells shrink dramatically in size. Growth of the secretory cell increases throughout the subsequent intermolt and

continues until the next molt. At the larval-pupal molt, dermal glands undergo programmed cell death.

Since their discovery (Verson, 1890), these giant cells of the dermal glands have been assumed to function in secreting a substance at the time of the molt– such as molting fluid (Barbier, 1970) or a cement layer (Horwath and Riddiford,1988; Wigglesworth, 1947) - that is discharged onto the surface of the cuticle. Secretory cells of dermal glands had therefore always been assumed to discharge the contents of their large vacuoles through the associated saccule and duct cells.

Lai-Fook (1973) observed that while the smaller saccule cell of the dermal gland clearly releases granules to the cuticular surface through the channel of its contiguous duct cell, the larger secretory cell has no evident structure resembling a secretory apparatus. Her detailed observations of larval secretion and discharge are not consistent with the dermal secretory cells having a function in molting as was suggested by Wigglesworth (1947), Way (1950) and Barbier (1970) and as subsequently assumed by Horwath and Riddiford (1988).

Despite all these earlier claims about functions for these large cells, Delhanty and Locke (1990) cautiously observed that "the function of the secretion is still uncertain". New information on the sequences of proteins that are secreted at the molt by *Manduca* dermal secretory cells provides clues about the function of the secretion and reinforces the claim that secretion from the vacuoles of the large secretory cell is discharged into the

surrounding hemocoel and internal larval environment rather that to the external environment and surface of the integument.

Materials And Methods

Rearing of larval *Manduca sexta*

All developmental stages of this insect were fed an artificial diet and maintained in an incubator at constant temperature (26°C) and constant photoperiod (16L:8D).

Preparation of whole mounts for lectin and antibody labeling

For dissections, anesthesized larvae were placed in petri dishes in which black Sylgard (Dow Corning) had been added as a substrate. To this silicone surface, whole first and second instar larvae were pinned ventral surface up with stainless steel minutien pins (0.1 mm diameter) and dissected in sterile Grace's insect culture medium (Invitrogen, pH adjusted to 6.5). Each cylindrical larva was cut along its ventral midline with iridectomy scissors from head capsule to anus. The cut edges of the larval integument were spread and pinned down, with two pins at the anterior end and two pins at the posterior end. Converting the initially cylindrical integument to a rectangular planar integument exposes the internal alimentary canal and ventral nerve cord. After excising the entire gut and nerve cord, the dermal cells, muscles, dorsal vessel and fat body that line the inner surface of the dorsal integument are exposed for viewing. After dissection, tissues were either processed for (1) sectioning or for (2) preparation of whole mounts. Upon addition

of fixative, the pinned tissue retained its planar configuration. Either whole larvae were labeled as whole mounts or specific regions were excised for sectioning or labeling.

Tissues were fixed for 30 min with 4% paraformaldehyde that had been dissolved in phosphate-buffered saline (PBS, pH 7.4). After three rinses in PBS, tissues were permeabilized for at least 30 min by the addition of blocking buffer (PBS + 10% normal goat serum + 0.1%Triton X-100). After an overnight incubation with PNA lectin coupled to either rhodamine or fluorescein (10µg/ml, Vector Laboratories) or with primary antibody (mouse anti-lacunin or mouse anti-neuroglian, 1:2,000) dissolved in blocking buffer at 4º C, tissues were rinsed at least three times with blocking buffer. After labeling with primary mouse antibodies, rinsed tissues were incubated overnight in the cold with 7.5 mg/ml of a secondary fluorescein isothiocyanate (FITC) coupled goat anti-mouse antibody (Vector). The two primary antibodies are mouse monoclonals prepared against the *Manduca* proteins neuroglian and lacunin (Nardi et al., 1999; Nardi, 1994). In addition, a specific marker for DNA was sometimes used to label nuclei of cells; its labeling concentration was 1 µg/ml. This specific DNA marker is a blue fluorescent compound known as 4',6-diamidino-2-phenylindole or DAPI. Following three more rinses with blocking buffer, labeled tissues were mounted in 70% glycerin (v/v) in 0.1 M Tris (pH 9.0). Fluorescently labeled specimens were imaged with a Nikon E600.

Preparation of Tissues for High-Resolution Microscopy

For high resolution imaging of the internal structures, whole dermal secretory cells or abdominal segments or hemisegments containing one or more dermal secretory cells were fixed in the cold for three hours with a mixture of 0.5 % paraformaldehyde and 2.5 % glutaraldehyde in a rinse buffer (0.1 M cacodylate buffer (pH 7.4) containing 0.18 mM $CaCl_2$ and 0. 58 mM sucrose). After this initial fixation, tissues were washed three times with rinse buffer before being post-fixed for three – four additional hours in the cold with rinse buffer containing 2 % OsO_4. Three additional washes with rinse buffer followed the post-fixation. To enhance membrane contrast, tissues were placed in filtered, saturated uranyl acetate for 30 min immediately prior to being passed through a graded series of ethanol concentrations (10% -100%).

From absolute ethanol, tissues for sectioning were transferred to propylene oxide and infiltrated with mixtures of propylene oxide and resin before being embedded in pure LX112 resin. Resin was polymerized at 60º C for three days followed by an additional overnight treatment in an 80º C oven.

Embedded tissues were sectioned with a diamond knife either at 0.5 µm for light microscopy or at ~0.09 µm for electron microscopy. Sections for light microscopy were mounted on glass slides and stained with a solution of 0.5% toluidine blue in 1% borax. Thin sections of those regions chosen for ultrastructural examination were mounted on copper grids and stained briefly with saturated aqueous uranyl acetate and Luft's lead

citrate to enhance contrast. Images were taken with a Hitachi 600 transmission electron microscope operating at 75 kV.

Processing of dermal secretory cells for electrophoresis

Two samples of dermal secretory cells were mailed to Kendrick Laboratories in Madison, Wisconsin for two-dimensional (2D) electrophoresis of deglycosylated proteins. One sample contained 61 pre-molt cells; the second sample contained 66 post-molt cells. To each sample was added 50 µl of SDS Boiling Buffer without reducing agents in addition to 50 µl of Osmotic Lysis Buffer with 10X nuclease stock, phosphatase inhibitor I and II stocks, and protease inhibitor stock. These samples were vigorously vortexed and heated at 100°C for 5 minutes. Concentrations of proteins were measured with the BCA (BiCinchoninic Acid) assay (Pierce Chemical Co., Rockford, IL). From each sample, an aliquot of 200 µg was deglycosylated with Enzymatic DeGlycoMix Kit (QA Bio). The procedure was carried out at 37°C for 3 h. The two lyophilized samples were finally dissolved in 1:1 diluted SDS Boiling Buffer: Urea Sample Buffer containing reducing agents prior to loading 200µg/50µl of each on gels.

A third sample of 20 dermal secretory cells was mailed to Kendrick Laborartories for lectin (PNA) blotting following 2D electrophoresis of glycosylated proteins. This sample was prepared for electrophoresis as described in the preceding paragraph but without the 3-hour deglycosylation procedure.

Two-dimensional electrophoresis

Two-dimensional electrophoresis was performed according to the carrier ampholine method of isoelectric focusing (IEF)(Burgess-Cassler et al., 1989). Focusing was carried out in a glass tube with an inner diameter of 2.3 mm using 2% pH 3-10 Isodalt Servalytes (Serva, Heidelberg, Germany) for 9600 volt-hours. An internal standard of tropomyosin added to each sample migrated as a doublet with lower polypeptide spot of MW 33,000 and pI 5.2. After equilibration in the following buffer (10% glycerol, 50mM dithiothreitol, 2.3% SDS, and 0.0625 M Tris, pH 6.8), each tube gel was sealed to the top of a stacking gel overlying a 10% acrylamide slab gel (0.75 mm thick). Protein separation in the slab gel occurred over a 4-h period at 15 mA/gel. Six molecular weight standards ranging between 220,000 kDa and 14,000 kDa marked the basic edge of each slab gel.

Lectin blotting

Duplicate gels for blotting were placed in transfer buffer (10mM Caps, pH 11.0, 10% methanol) and blotted overnight to a PVDF membrane (200 mA, ~100volts/two gels) using the same six molecular weight standards listed in the above paragraph. After staining with Coomassie Brilliant Blue R-250, PVDF membranes were scanned and destained in 100% methanol. A rinse in Tween-20 Tris-buffered saline containing 0.01 mM Mn^{+2} ($TTBS^{++}$) preceded blocking of the membranes in 5% bovine serum albumin (BSA) for two hours. The blots were incubated overnight in biotinylated peanut agglutinin (PNA, Vector Laboratories) that had been diluted in 2% $TTBS^{++}$. After three rinses in $TTBS^{++}$, the two blots were incubated for two hours with poly horseradish peroxidase (HRP) Streptavidin (Thermo) diluted 1:50,000 in 2% BSA -$TTBS^{++}$.

Following three rinses with TTBS^{++}, blots were treated with enhanced chemo-luminescence (ECL) substrates and exposed to X-ray film.

Preparation of tissues for mass spectrometry

Preliminary examination of specific proteins from these exceptionally large cells involved extraction and clean-up of total protein from ten entire pre-molt secretory cells as a prelude to mass spectrometry analysis of the prominent proteins. Excellent matches were observed for the cytoskeletal proteins actin, non-muscle myosin II, tubulin, spectrin, and moesin. Heat shock protein 70 consistently appeared as one of the more abundant proteins of these cells.

Protein sample extraction and preparation for trypsin digestion and liquid chromatography/mass spectrometry (LC/MS)

Proteins from dermal secretory cells were collected and extracted using Minute^{TM} Total Protein Extraction Kit (Invent Biotechnologies, Inc. Eden Prarie, MN) following manufacturer's protocol. Briefly, up to 20 mg of tissue were ground using a disposable plastic pestle and extracted using 200 microliters of denatured lysis buffer. The protein extract was cleaned up using Perfect Focus^{TM} (G-Biosciences, St. Louis, MO) and processed through Pierce C-18 Spin Columns (Rockford, IL). The resultant protein was lyophilized and digested with Trypsin MSG (G-Biosciences, St. Louis, MO) at a ratio (w/w) of 1:20 in a CEM Discover Microwave Digestor (Matthews, NC) at 55° C for 30 minutes. The digested peptides were analyzed using a Thermo-Dionex Ultimate RSLC3000 nano UHPLC and a Thermo LTQ Velos pro ETD mass spectrometer.

Protein digestion and peptide isolation

Coomassie Blue-stained protein spots were excised with a scalpel and washed in water (HPLC grade). All subsequent steps were performed in 50 mM ammonium bicarbonate. The gel pieces were dried in a vacuum centrifuge and then rehydrated in ammonium bicarbonate. This was repeated two times. Finally, the gel pieces were rehydrated in ammonium bicarbonate +10 mM dithiothreitol (DDT) and incubated at 56°C for 45 min. The DTT solution was replaced with ammonium bicarbonate +100 mM iodoacetamide and incubated for 45 min in the dark with mixing. Then, the gel pieces were re-incubated with mixing in ammonium bicarbonate in 50% acetonitrile, followed first by 100% acetonitrile and then by drying in a vacuum centrifuge. The dry gel pieces were rehydrated in ammonium bicarbonate with 10 ng/µl trypsin and incubated overnight with low shaking at 37°C. Peptides were extracted from the gel pieces two times with 5% formic acid/ammonium bicarbonate/50 % acetonitrile and once with 100% acetonitrile. The resulting peptide-containing supernatants were pooled and dried in a vacuum centrifuge before being solubilized in 20 µl of 0.1 % formic acid/2% acetonitrile.

LC-MS/MS

LC-MS was performed using a Thermo Dionex Ultimate RSLC3000 (Thermo Scientific) operating in nano-flow mode at 300 nanoliters/min with a gradient from 0.1% formic acid to 100% acetonitrile + 0.1% formic acid in 120 minutes. The trap column used was a

Thermo Acclaim PepMap 100 (100 µm x 2 cm) and the analytical column was a Thermo Acclaim PepMap RSLC (75 µm x 15 cm). The eluent from the column was sprayed directly into a Thermo Velos Pro LTQ mass spectrometer running a triple play method consisting of a survey scan, followed by up to five data dependent zoom and MS/MS scans on the highest intensity peaks in the survey scan.

Mass spectral data analysis

Xcalibur raw mass spectral chromatogram files were converted by Mascot Distiller (Matrix Science) into peak lists that were submitted to an in-house Mascot Server and searched against specified protein databases. Only individual ion scores with p less than or equal to 0.05 were used to determine positive protein hits.

Results

Description of these giant cells in *Manduca sexta*

Tucked between the integument and muscles lie the largest cells of the larval body - secretory cells of dermal glands. In their cloistered, unexposed locations, these cells are easily overlooked despite their large sizes. Dermal glands exist as pairs in each thoracic segment and nine abdominal segments (Fig. 1a,b). Fig. 1c shows Nomarski images of a section through a pair of these glands in the dorsal region of the 3rd abdominal segment of a third instar larva immediately before ecdysis.

Labeling whole mounts of first instar larvae with the lectin peanut agglutinin (PNA) facilitates this visualization of the global organization of these cells. This lectin intensely

labels the contents of the secretory cell's numerous vacuoles in Fig. 1b and conveniently facilitates tracking of the release of the vacuoles' contents and their subsequent restoration during each molt cycle.

The secretory cell is the largest of the three cells that make up the dermal gland (Figs. 2b-d), growing to lengths of several millimeters in mature larvae. The vacuoles of the cell in Figs. 2a,b label intensely with PNA-rhodamine, and nuclei label with the blue fluorescent stain DAPI. The exceptionally large secretory cell has a highly convoluted, labyrinthine nucleus (Fig. 2c). The extensive surface area of this fenestrated, polyploid nucleus is accompanied by an equally convoluted and highly ramified endoplasmic reticulum.

Secretion from dermal secretory cells

Secretion of the contents of the numerous vacuoles occurs abruptly at the time of the molt. Lectin labeling and ultrastructural examination of the secretory cells and their associated saccule and duct cells at different developmental stages have provided no evidence for the contents of the numerous vacuoles of the secretory cells being secreted through the integumentary duct cell. The duct and saccule cells are separated from the larger dermal secretory cell by an uninterrupted cell membrane. The secretory duct terminates in the saccule cell (Fig. 2b). Although the cuticular lining of the smaller saccule cell is contiguous with the surface of the integument, this study provides no evidence that the much larger dermal secretory cell expels any of its contents on the cuticular surface of the overlying integument.

Immediately after the molt and during most of the intermolt, the vacuoles of the dermal secretory cell no longer label with PNA. Lectin labeling of the dermal secretory cells is not obvious again until shortly before the molt. At the molt, the vacuoles rapidly shrink in diameter. Two hours before the molt, surfaces of vacuoles are smooth (Fig. 3a). As the contents of the vacuoles are released at the molt, however, fine membrane-bound processes measuring about 70-80 nm in width dramatically increase in density as they extend into each vacuole from the vacuole's steadily shrinking periphery (Figs. 3b-d). This periphery becomes increasingly electron-dense as the vacuole shrinks in size. As the perimeters of the vacuoles shrink, the spacing between these processes that line the lumens of the vacuoles concomitantly decreases. By the time the molt is complete, the interior of each vacuole is filled with numerous processes and highly convoluted membranes (Fig. 3e,f).

No obvious connection exists between the large vacuoles and the extracellular environment. Cytoskeletal elements line the outer surfaces of these large vacuoles, and a well-defined electron-dense band circumscribes each contracting vacuole (Figs. 3b-f). High densities of cytoskeletal filaments are concentrated on the outer surfaces of shrinking vacuoles that were fixed immediately after a molt (Fig. 3f) and as observed by Delhanty and Locke (1990).

As the vacuoles and the entire secretory cell shrink after each molt (Fig. 4g), numerous vesicles appear for the first time around their circumferences (Figs. 3b-d, 4h,i). As the amorphous contents of the vacuoles are emptied from the dermal cells and are replaced by numerous fine processes that extend centripetally (Fig. 3b-f), these smaller vesicles

arranged centrifugally around emptying vacuoles concomitantly fuse with the surface of the dermal cell and discharge into the extracellular environment (Figs. 3b-e, 4h,i). As the contents of the depleted vacuoles are restored during the intermolt period, however, a reversal in the centripetal extension of fine processes associated with evacuation of the vacuole occurs. The 70-80 nm processes lining the inner surfaces of the vacuoles dramatically decrease in number and density as the contents of vacuoles are restored during the intermolt (Figs. 4j,k). The membrane-bound processes that had extended centripetally during the molt (Figs. 3b-f) begin retracting prior to each molt until the inner surface of each vacuole has returned to a smooth configuration (Fig. 3a).

Analysis of proteins secreted by dermal secretory cells

The ability to separate cleanly individual dermal secretory cells from their surrounding cellular environments has enabled identification of proteins that are expelled from these cells at the molt from fourth larval instar to fifth larval instar. Dermal secretory cells from the 7^{th} and 8^{th} abdominal segments were collected from late fourth instar larvae at 2 hours before their upcoming molt (pre-molt cells). These larvae were precisely staged by the condition of their head capsules. At this stage in the molt cycle, the cuticle of the 4^{th} instar head capsule has retracted from the underlying newly formed 5^{th} instar head capsule. Corresponding dermal secretory cells (post-molt cells) were dissected from early 5^{th} instar larvae about one hour after their previous molt. By running and comparing 2D gels of proteins extracted from these two discrete stages, those proteins present immediately prior to the molt but absent immediately after the molt can be readily

distinguished from those housekeeping proteins that are shared by dermal secretory cells both before and after a molt.

Since heavily glycosylated proteins often present sequencing challenges, 2D electrophoresis was carried out on a pair of gels from protein samples that had been deglycosylated. By comparing pre-molt proteins in dermal secretory cells before a molt (Fig. 5a) with those remaining in dermal secretory cells after a molt (Fig. 5b), spots of proteins that are expelled from these secretory cells can be readily identified. Proteins secreted at the molt are absent from the post-molt gel but stand out in the pre-molt gel. Six of these secreted proteins (D1-D6) were marked in Fig. 5a, excised from the Coomassie blue-stained gel, and subsequently submitted for mass spectrometry (Table 1).

An initial blot from a 2D gel of pre-molt dermal secretory cells whose proteins had not been deglycosylated (Fig. 5c) was probed with biotinylated peanut agglutinin (PNA). PNA is a marker for proteins of the secretory cell vacuoles but does not necessarily label all proteins that are secreted from these vacuoles. However, one protein spot (G3) recognized by the PNA lectin was excised from this initial 2D gel and identified by mass spectrometry as the same protein that later was identified as a member of the SERPIN superfamily (D2) from the gels shown in Figs. 5a,b. Two other proteins excised from this gel (G1, G2) provided sequence information. These novel proteins are members of the Nitrilase and Major Royal Jelly Protein superfamilies; these proteins are known to be heavily glycosylated proteins. The proteins from this 2D gel of glycosylated proteins are listed in Table 1.

Discussion

The long-held view that dermal secretory cells discharge the contents of their large vacuoles onto the surface of the newly forming cuticle at each molt has been challenged by new evidence. Analysis of contents of these vacuoles suggests that proteins from secretory cells represent components of an innate immune response rather than components of molting fluid or newly formed cuticle. During the vulnerable molting periods in insect lives, secretion of humoral components of innate immunity would provide enhanced protection from foreign invaders.

A distinctive secretory process

Secretion of the contents of the numerous vacuoles of the dermal secretory cells occurs abruptly at the time of the molt. Lectin labeling indicates that the contents of the vacuoles are expelled into the surrounding hemocel and not into the contiguous saccule cell. Ultrastructural inspection of the secretory cells during this relatively brief period reveals that these vacuoles do not directly expel their contents by exocytosis. Rather the secretory mechanism involves contraction of each vacuole's perimeter.

As the vacuoles shrink, numerous vesicles appear for the first time around their circumferences (Figs. 3b-e, 4h,i). These vesicles presumably represent the route by which the contents of vacuoles are expelled from the cells. As contents of the vacuoles are emptied from the dermal cells (Figs. 3b-f), these vesicles concomitantly fuse with the surface of the dermal cell and discharge into the extracellular environment

Certain vertebrate immune secretory cells of the myeloid lineage also display such a distinctive secretory mechanism and in this respect represent counterparts of the dermal secretory cells of insects. Eosinophils, neutrophils, and mast cells are immunoregulatory leukocytes that are involved in very rapid extracellular release of the contents of their numerous granules during inflammatory responses (Spencer et al., 2014). Conventional exocytosis of granules and/or vacuoles by their fusion with the plasma membrane and subsequent release of their entire contents does not typically occur in these cells. The contents of granules and vacuoles are expelled in a stepwise fashion with contents being transferred to smaller vesicles before release at the plasma membrane. This distinctive secretion mechanism involving vesicular transport of small aliquots of proteins from granules and vacuoles to the cell surface is referred to as "piecemeal degranulation".

Characterization of proteins secreted by dermal secretory cells

Many proteins such as those secreted by the giant dermal cells are not only multifunctional but can also influence a process directly or indirectly through their interactions with other proteins. All the proteins listed in Table 1 represent novel proteins; these proteins may be produced only in the dermal secretory cells and in no other tissues. Although their exact functions remain unknown, their domain structure places them in particular superfamilies whose general roles have been characterized. A subset of the proteins in Table 1 are known to represent insect protein groups with well documented functions in innate immune responses (Kanost and Nardi, 2010). These are Serine Proteinase Inhibitors (SERPINs), C-type lectins, GMC oxidoreductases, and members of Major Royal Jelly Protein superfamily (Zhang et al., 2014).

Members of the SERPIN superfamily are proteinase inhibitors involved in regulating activity of the multiple proteinases that initiate biochemical cascades such as the prophenoloxidase activation pathway during immune responses and development. These include spot D2 (from gel of deglycosylated proteins) and spot G3 (from gel of glycosylated proteins).

Spot D3 represents a protein in the C-type lectin superfamily. These lectins are often referred to as immulectins and have been shown to act as Pattern Recognition Receptors or PRRs that recognize the conserved molecular patterns on surfaces of microbial cells (Yu et al., 2006).

Members of the GMC oxidoreductase superfamily such as FAD-glucose dehydrogenase have been shown to be induced during cell-mediated immune responses in *Manduca* and are believed to interact with the phenoloxidases that are associated with recognition of nonself in insect hemolymph (Cox-Foster and Stehr, 1994). Two different members of this superfamily (spot D1 , 41,000 kDa and spot D5, 29,000 kDa) are secreted at the time of the larval molt.

Spot D4 represents a member of the carbonic anhydrase superfamily. In addition to influencing the acid-base balance of tissues, carbonic anhydrase IX deficiency influences immune and defense responses in gastric epithelium of mice (Kallio et al., 2010).

Members of the Major Royal Jelly Protein (MRJP) subfamily are N-glycosylated proteins that perform a variety of functions throughout development in different tissues (Drapeau

et al., 2006). These proteins have antibacterial activity and are noteworthy for their potent immunoregulatory activity in mice (Zhang et al., 2014).

The sequence of one of the secreted proteins (G1) corresponds to a biotinidase-like member of the nitrilase superfamily. These are typically highly glycosylated proteins. Deficiencies in biotinidases prevent recycling of biotin from the many enzymes to which it is bound; enzymes such as essential carboxylases cannot function in its absence (Wolf and Jensen, 2005).

Spot D6 represents an acidic deglycosylated protein with no putative conserved domains. However, close inspection of this cationic polypeptide with 107 amino acid residues shows that it has all the hallmarks of an antimicrobial peptide (Tassanakajon et al., 2014).

Conclusion

Each molt represents a time of vulnerability for an animal. With the newly forming cuticle still thin and soft and the mobility of the animal compromised, responding cautiously to this vulnerable condition seems to be a good survival strategy. Secretion of novel proteins, some or all of which could function as immune-regulatory proteins, from these giant cells of the dermal glands at the time of each molt would help ensure safe passages between larval stadia. Inclusion of dermal glands to the repertoire of insect defenses adds a new and unsuspected layer of complexity to the insect immune system.

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Figure Legends

Fig.1. Dermal secretory cells lie within the larval hemocoel.

a. In dorsal view of a mature fifth instar larva, 12 pairs of dermal secretory cells (labeled green) are symmetrically arranged around heart (H). Bar = 10 mm.
b. Interior view of a first instar larva whose gut and neural cord were removed after being opened along its ventral midline. The dermal secretory cells, brain (arrowhead) and hematopoietic organs (arrows) have been labeled with PNA-FITC. The tracheal tubes are lined with cuticle and show green autofluorescence. Anterior is to the right. Scale bar = 1.0 mm.
c. After labeling 4th abdominal segment of a mature third instar larva with neuroglian-HRP, tissue was fixed and sectioned. In this section viewed with Nomarski optics, labeled secretory cells (arrows) and their large vacuoles stand out among surrounding larval epidermis (e), muscles (m), fat body (fb) and heart

(h). Cuticle of fourth instar has formed on larval epidermis (e); cuticle of third instar (3c) has undergone apolysis but not ecdysis.

Fig. 2. Dermal secretory cells at 2 h before a molt were prepared as whole mounts.

a. A view of a 7th abdominal hemisegment of a mature third instar imaged *in situ* after being labeled with an antibody (anti-lacunin-FITC) that recognizes basal laminae of muscles and granular hemocytes. The dermal secretory cell was labeled with PNA-rhodamine. Region of saccule and duct cell attach-ment to larval integument is indicated with an arrowhead. Bar = 100 µm.

b. This dermal secretory cell and associated saccule cell (s) and duct cell (d) were dissected with a patch of overlying 4th instar larval integument (i). These cells were labeled with PNA-rhodamine and DAPI. The larval integument exhibits green autofluorescence. Bar = 200 µm.

c. This specimen is identical to the previous specimen, but only blue fluorescence of DAPI is shown in grayscale. The polyploid spongiform nucleus of the secretory cell dwarfs the small nuclei of the larval integument (i) and the U-shaped polyploid nucleus of the saccule cell (arrowhead).

d. Diagram of a dermal secretory cell one hour before a molt shows salient features. sec = secretory cell; sac = Saccule cell; D.c. = duct cell; L.i. = larval integument; n = nucleus; v = vacuole.

Fig. 3. Ultrastructural features of dermal secretory cells reveal the structural changes in vacuoles immediately before and during the 4th to 5th larval molt. Portions of spongiform

nucleus (n) are labeled. A basal lamina (bl) covers the surface of the cell. Vacuoles =v. Scale bars = 1 µm (d, e, f); 5 µm (a); 10 µm (b,c).

a. Two hours before molt (4^{th} to 5^{th} instar), vacuoles are filled with amorphous matrix.
b. At the molt, processes (arrowheads) extend into vacuoles,
c. increasing in number and density as vacuole shrinks.
d. At electron-dense interface between vacuole and cytoplasm of the cell (arrows) , vesicles (ve) appear on cytoplasmic side.
e. At completion of vacuole collapse, processes and membranes (m) fill interior of vacuole. Vesicles (ve) surround vacuole perimeter (arrows).
f. Cytoskeletal elements (ce) lie between processes (p) of collapsing vacuole periphery (arrows) and surrounding cytoplasm.

Fig. 4. After the 4^{th} to 5th larval molt, the vacuoles are replaced by smaller, even more numerous vesicles as proteins are expelled into the hemolymph. The vacuoles then refill during the intermolt. Portions of spongiform nucleus (n) and basal lamina (bl) are labeled. Scale bars = 1 µm (k); 10 µm (h,i,j); 1 mm (g).

g. Sections of two dermal secretory cells provide a global view of cells whose vacuoles have been emptied. These cells fail to label with PNA.
h. The once smooth cell surface is now convoluted; small vesicles occupy space between cell's surface and nucleus (n).
i. Vesicles and cell membrane fuse.

j. As vacuole contents are restored during intermolt, processes (arrowheads) that earlier filled vacuoles shrink in number and density.

k. In this intermolt cell, two processes (arrowheads) extend about 0.7µm into vacuole.

Fig. 5 a, b. Two-dimensional gels of deglycosylated proteins from (a) pre-molt dermal secretory cells are compared with deglycosylated proteins from (b) post-molt dermal secretory cells. Specific spots disappear from dermal secretory cells at the time of the molt from 4^{th} instar to 5^{th} instar larva. These spots have been circled and numbered D1-D6 in (a). Each of these spots was excised and submitted for identification by mass spectrometry.

Fig. 5c. Two-dimensional gel of glycosylated proteins from pre-molt dermal secretory cells. This gel was subsequently blotted with PNA-biotin + strepavidin-HRP to reveal protein spots recognized by the PNA lectin. Three of the labeled spots (G1-G3) were chosen for mass spectrometry.

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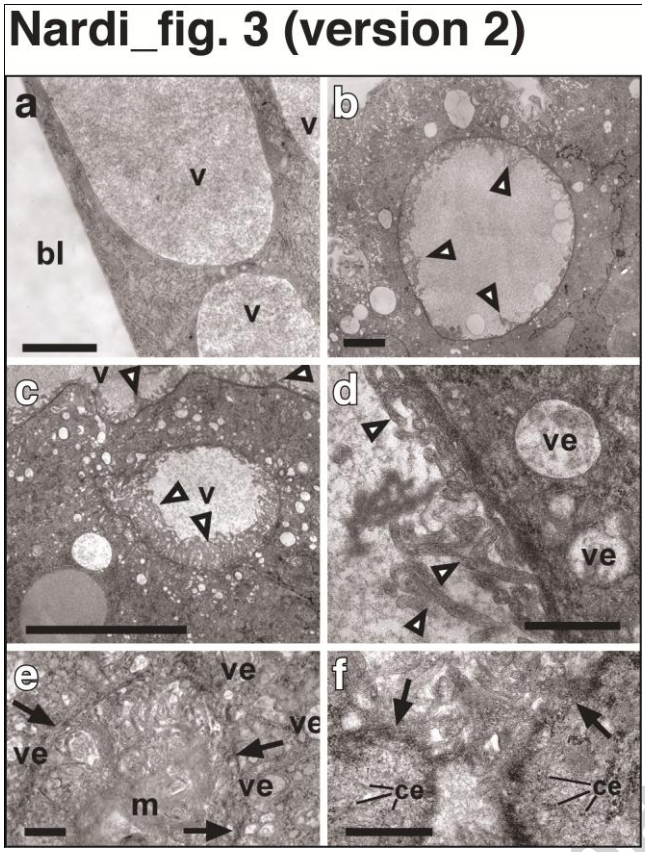
Table 1. Listing of proteins secreted by dermal secretory cells and separated by 2D electrophoresis. In the first column, G designates spots from the gel of glycosylated proteins; D designates spots from the gel of deglycosylated proteins. Identification value for proteins was set for $p<0.05$.

Spot number and *approximate molecular weight* (*kDa*)	Accession number in *Manduca sexta* protein database	Protein description based on conserved domains
G1, *62*	12942	Nitrilase superfamily, biotinidase like
G2, *45*	03572	Major Royal Jelly Protein/Yellow superfamily
G3, *42*	10824	SERPIN superfamily

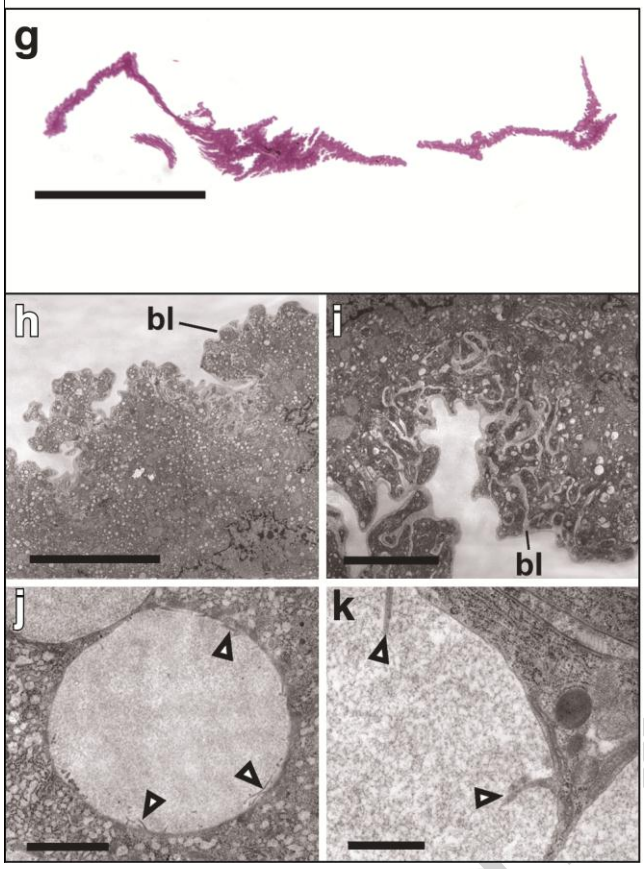
D1, *60*	11723	NADB_Rossmann superfamily GMC_oxred_C superfamily
D2, *41*	10824	SERPIN superfamily
D3, *31*	01104	C-type lectin superfamily
D4, *30*	04279	alpha_Carbonic anhydrase superfamily
D5, *29*	11789	NADB_Rossmann superfamily NAD(P)_dependent dehydrogenase
D6, *11*	07541	No putative conserved domains

Highlights

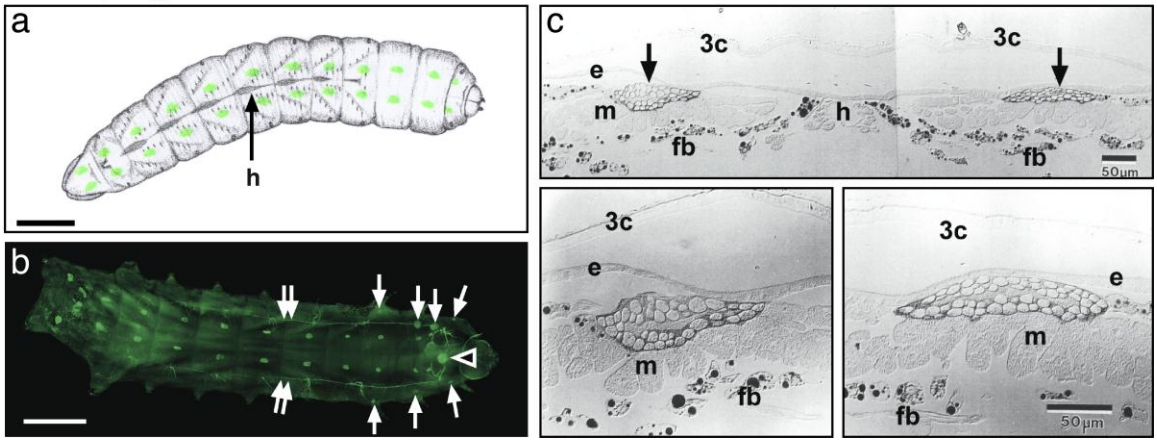
- A pair of dermal secretory cells occupies all thoracic and nine abdominal segments.
- The lectin peanut agglutinin intensely labels contents of secretory cell vacuoles.
- Contents of vacuoles are abruptly expelled at each molt.
- Vacuoles discharge contents by a distinctive piecemeal process.
- Novel proteins secreted by secretory cells have presumptive immune functions.



Nardi_fig. 4 (version 2)



Nardi_fig. 1



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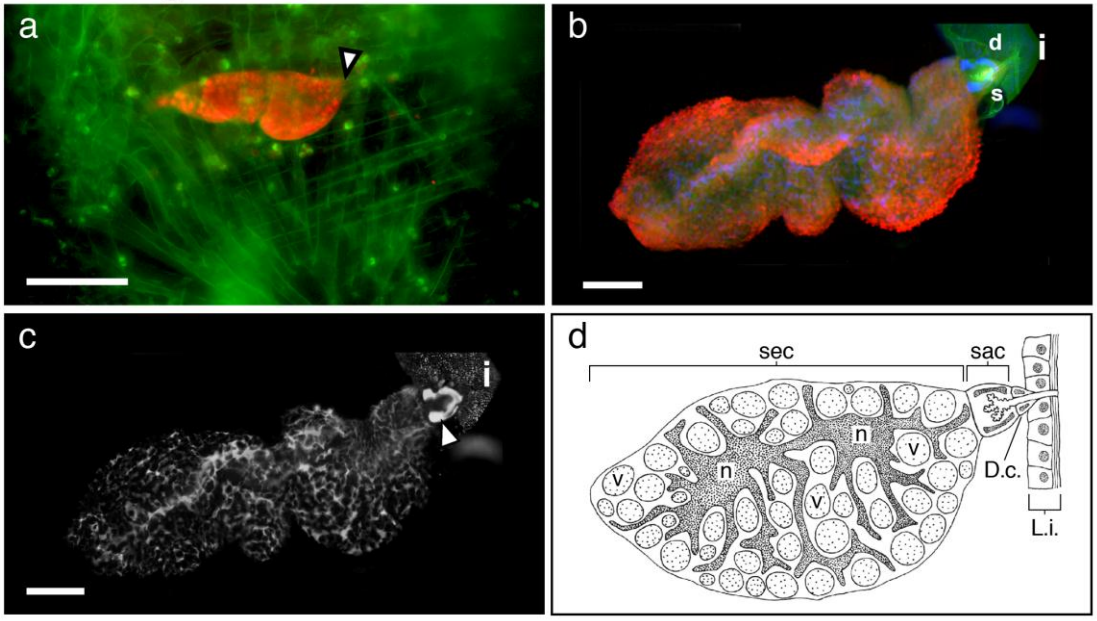
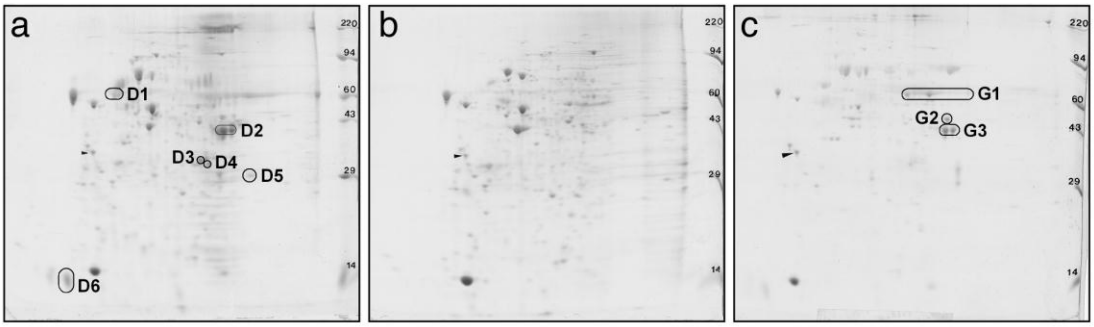
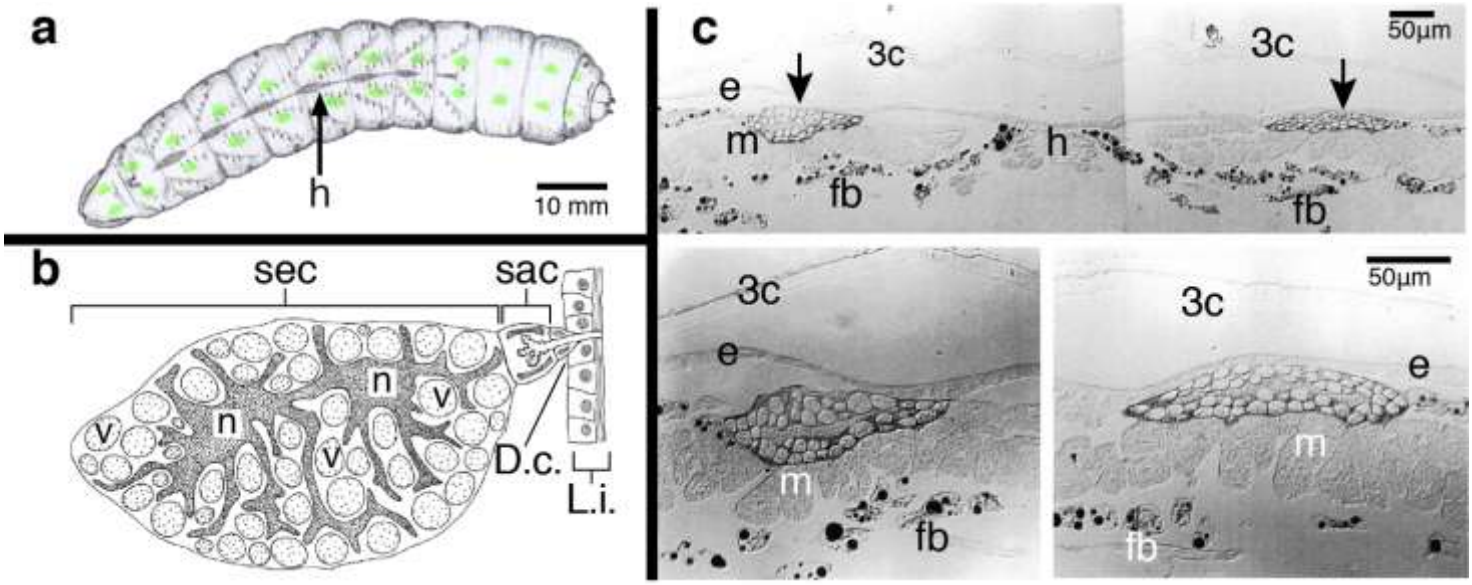


Nardi_fig. 5





(a) Pairs of dermal glands (green) lie in 12 segments on dorsal surface of mature larva. h= heart. **(b)** Each gland attaches to larval integument (L.i.) and consists of a secretory cell (sec), saccule cell (sac) and duct cell (D.c.). n = nucleus; v = vacuole. **(c) Top**: In a section of 3rd instar larva just before a molt, a pair of secretory cells (arrows) are symmetrically arranged around the heart (h). **Bottom,** close ups: 3rd instar cuticle (3c) has retracted from epidermis (e) but has not yet been shed. m = muscle; fb = fat body.