Ultrastructure of the spines and neck gland of Abananote hylonomo Doubleday, 1844 (Lepidoptera: Nymphalidae)

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Abstract

The external morphology of the cuticular spines, and the ultrastructure of the spines and neck gland in fifth instar Abananote hylonomo larvae was studied. The larvae are spiny along the length of their bodies. Along the length of the spines are setae with a swelling towards the apical region. Internally, in the base of each seta there is a complex of secretory cells surrounding a large vacuole continuous with the seta. The neck gland is eversible, composed of a pair of oval internal sacks connected to the exterior via an extracellular canal produced by an invagination of the cuticle. The sack cells surround a reservoir containing an amorphous substance. In both the spines and neck gland the nuclei are large and irregularly shaped, typical of defensive glands of Lepidoptera. The border of the cells adjacent to the vacuoles (spines) and the reservoir (neck gland) is made up of numerous microvilli. We suggest that defensive compounds are produced in the gland cells and then later released via the vacuoles in the spines and the extracellular canal in the neck gland. © 2000 Elsevier Science Ltd. All rights reserved.

1. Introduction

Lepidopteran larvae are considered an attractive prey due to their soft cuticle and relative immobility. Nevertheless, many of these larvae have developed stinging hairs, spines and glands which secrete noxious chemicals in order to ward off both vertebrate and invertebrate predators.

Abananote hylonomo is a Nymphalid butterfly, which in its larval stage lives gregariously on its hostplant, Verbania sp. (Compositae). The larvae are spiny from the third instar onwards, and all instars possess an eversible neck gland, situated ventrally in the thorax, just anterior to the first pair of legs.

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Behavioural experiments demonstrate that both the spines and the neck gland contain substances repellent to at least two ant species, Camponotus rufipes and Solenopsis geminata. Chemical analysis has shown that these substances include several carboxylic acids (Osborn and Jaffe, 1998). Further behavioural experiments proved that two of these acids, linoleic acid and oleic acid are responsible (at least in part) for the repellency of the larvae (Osborn and Jaffe, 1998).

With respect to the glands contained in larval spines, Gilmer (1925) described two basic types; simple spines and specialised spines. Simple spines consist of an ordinary seta with a gland cell in its base that secretes toxic substances towards the lumen of the seta. Specialised spines are similar in that the stinging apparatus is also a seta, but this seta is located at the end of a multicellular spine formed by an evagination of the epithelium (Gilmer, 1925; Snodgrass, 1993). Although species of caterpillars with toxic spines are

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known from several Lepidopteran families, including the Nymphalidae, there have been few detailed studies of their ultrastructure (Kawamoto et al., 1978; Novak and Lamy, 1987).

Regarding the neck gland, the only studies of the ultrastructure have been undertaken with larvae from the Notodontidae (Hintze, 1969; Percy and MacDonald, 1979; Weatherston et al., 1986). Nevertheless, these glands are also characteristic of larvae from the Nymphalidae (subfamilies Satyrinae and Brassoilinae) and other taxa from this family (DeVries, 1987).

Here we study the ultrastructure of the spines and neck gland in *Abananote hylonomone* in order to determine the existence of specialised glands responsible for the synthesis and release of the defensive compounds produced by this species in its larval stage.

2. Materials and methods

Fifth instar larvae of *Abananote hylonomone* were collected from their hostplants *Verbania* sp., found commonly in the campus of the University Simón Bolívar, Caracas, Venezuela. The larvae were dissected in distilled water using a stereoscopic microscope, Zeiss Stem SV 11. Whole sparks were cut off and the neck glands were removed by cutting the tissue surrounding them, and then gently lifting the gland with a pair of forceps. The dissected spines and neck glands were immediately prefixed in a 2.5% gluteraldehyde/1% paraformaldehyde solution, buffered with 0.1 M sodium cacodylate at pH 7.2, 260 mOsm/l at 4°C, then postfixed for 1 h in 1% OsO4 in 0.1 M sodium cacodylate at 4°C and pH 7.2. For scanning electron microscopy, specimens were further dehydrated using a graded acetone series and by critical-point drying in a Balzers CPD 020 with CO2. The samples were then covered with gold/palladium and observed in a Philips SEM 505 scanning electron microscope (Lu and Chow, 1991; Stobbeart and Shaw, 1964).

For transmission electron microscopy, specimens were dehydrated in a graded ethanol series using conventional methods, and infiltrated with a mixture of propylene oxide: Polybed 812 at room temperature for 2 h and embedded in a resin mixture Polybed 812 for 48 h at 60°C. Semithin sections (1 μm) were cut using a Reichert ultramicrotome with a diamond knife and stained with toluidine blue at 4% in borax and observed in a Polvar (Reichert) microscope. Ultrathin (60 nm) sections were cut using the same ultramicrotome and stained with 6% uranyl acetate and lead citrate. They were observed in a Philips (EM-400 T) transmission microscope, at an accelerating voltage of 80 kV.

3. Results

3.1. External structure of the spines

The larvae are spiny along the length of their body. The external structure of the spines is illustrated in Plate 1. The spines are filled with what appears to be tissue (Plate 1 (3)) and at irregular intervals along their length are setae which project outwards from the spine at angles of between 30 and 90° (Plate 1 (1 and 4)). The setae are inserted in the spines in sockets (Plate 1 (2)) and towards the apical region, a bulbous swelling can be observed (Plate 1 (1 and 4)).

3.2. Internal structure of the spines

The internal structure of the spines is illustrated in Plate 2. Setae are distributed along the spines in the form of bristles of variable size. In the base of each seta are clearly recognisable cellular structures which surround a large vacuole continuous with the lumen of the seta (Plate 2 (5)).

The nuclei within these cells are typical of defensive glands of Lepidoptera, being large and irregularly shaped (Plate 2 (6)) (Gilmer, 1925; Snodgrass, 1993). The chromatin within these nuclei appears as discrete clumps, among which a homogenus, granular material is found (Plate 2 (7)). The cytoplasm contains both rough and smooth endoplasmic reticulum (Plate 2 (7) and insert), and a high density of mitochondria, especially close to the vacuoles, where the number of lysosomes is also higher (Plate 2 (8)). In the regions of the cells close to the vacuoles, an epithelial junction complex can be seen (Plate 2 (8) insert). In this same insert, the presence of vesicles can also be observed, responsible for the intercellular transport of substances (pinocytosis). These vesicles are electron-dense and appear as the classic omega figure inserted in, or separate from the cell membrane. Where the gland cells come into contact with the vacuoles, the cell walls are made up of numerous microvilli (Plate 2 (8)).

In some of the cells the cytoplasm is denser, containing abundant free ribosomes. There are also mitochondria and small vacuoles, with the latter found towards the cell junction (Plate 2 (8)).

3.3. Structure of the neck gland

The neck gland is visible, composed of a pair of internal oval sacks, ±0.3–0.2 mm, formed by acine units (Plate 3 (9 and 10)). Each one of these sacks contains cells with a dense cytoplasm that surround a reservoir containing an amorphous substance. The nuclei are large and irregularly shaped, in the same way as in the glands associated with the spines (Plate 3
(9)). In the base of each sack an extracellular canal joins up to a central canal, surrounded by cells, which immediately opens out to the exterior (Plate 3 (9 and 10)).

3.4. Ultrastructure of the sack cells

The cells that make up the sacks are delimited by a basal membrane, observed as a homogeneous material,
(16) Detail of one of the cells surrounding the Canal. C: Canal, PC: epicuticle, NC: endocuticle, Mv: microvilli, Ri: ribosomes, M: mitochondria, ▲ electron dense substance in the apexes of the microvilli. Bar = 0.3 μm. (TEM).
0.2–0.4 μm thick (Plate 3 (11)). The basal surface of the cells next to this membrane is highly folded and appears as fingers pointing towards the membrane, thus increasing the area for a greater absorption of material (Plate 3 (11)). In some of the sections, there is a high concentration of mitochondria close to the basal membrane, and immediately adjacent to these, packets of muscle fibre can be seen (Plate 3 (12)).

The cytoplasm of the cells is dense and contains a large number of mitochondria. Free ribosomes are found throughout the cytoplasm. (Plate 4 (13)). The nuclei are similar to those observed in the glands associated with the spines and within them the chromatin can be observed as discrete clumps, among which a homogeneous, granular material can be found (Plate 4 (13)). The cells are interdigitated and on their lateral sides, close to the reservoir, the occludens zone of the epithelial junctions can be observed (Plate 4 (14)). In the cytoplasm close to the reservoir, there is a complex of microtubules and the border of the cells is made up of microvilli (Plate 4 (14)). Note that where the extracellular canals lie adjacent to the cytoplasm no microvilli are present (Plate 4 (13)).

3.5. Ultrastructure of the canal cells

The cytoplasm of the canal cells contains mitochondria and free ribosomes but microtubules were not observed. The nuclei are irregularly shaped, as in the other cells described (Plate 5 (15)). The surface of the cell next to the cuticle contains numerous microvilli and in the apex of some of these there is a dense material (Plate 5 (16)).

4. Discussion

4.1. The spine cells

The cells found in the base of the setae in Ahabanote hylonomo are typical of cells of defensive glands in Lepidoptera, except for one of the cells where the dense cytoplasm suggests that this may be the trichogen cell responsible for the formation of the gland cell according to the description given by Gilmer (1925). The gland cells may be distinguished by their large size, and by the large and irregularly shaped nuclei (Gilmer, 1925; Snodgrass, 1993). The bases of the glands rest inside the spine, and thus correspond to a specialised spine type I (Gilmer, 1925). This type of spine has been reported in only a few families of Lepidoptera: Eucleidae, Megalopygidae, Noctuidae, Saturniidae and Nymphalidae (Eaton, 1988). The fact that the cytoplasm of the cells is dense and contains many mitochondria of uniform size, indicates that the cell utilises a large amount of energy (Eisner et al., 1964; Lu and Chow, 1991). The presence of smooth endoplasmic reticulum implies that the gland is a site for the production of non-protein substances. This agrees with the literature on the defensive chemistry in Lepidopteran larvae, given that the exocrine secretions reported for other species are also non-protein and include organic acids (Attygalle et al., 1993; Honda, 1983; Osborn and Jaffé, 1998; Percy and MacDonald, 1979), alcohols (Attygalle et al., 1993) and aromatic compounds (Deml and Dettner, 1994). The cellular structures described in this paper were also reported for the cells (in particular the tubular arm cell) that conform the defensive gland of Papilio spp. (Crossley and Waterhouse, 1969; Lu and Chow, 1991).

The defensive secretions of other Lepidopteran larvae may be stored in reservoirs, such as in the neck glands of Schizura unicornis and S. badia (Notodontidae), or synthesised in the moment of defence, for example, Papilio spp. (Crossley and Waterhouse, 1969; Lu and Chow, 1991). In the case of the spinal glands in Ahabanote hylonomo, the defensive compounds are probably stored in the vacuoles continuous with the setae, or even in the setae themselves. Thus the volatiles are synthesised in the cells and transported to the vacuoles by means of the numerous microvilli. These increase the surface area of the cell wall, and thus the rate of secretion of the compounds, which is to be expected in a defensive gland where the rapid liberation of the volatiles is of absolute necessity for an effective defence system.

Other morphological and ultrastructural studies have been undertaken of the spines and spicules of several urticating species of Lepidoptera (Kawamoto and Kumada, 1985; Kawamoto et al., 1978; Novak and Lamy, 1987; Novak et al., 1987). Nevertheless it is important to point out that in these cases both the mechanism of action and the target are different, contact being required for the release of the urticating substances, probably into the skin of vertebrate predators. In the case of A. hylonomo, the repellent substances are volatiles which are released into the air in order to repel attacking ants, and possibly other arthropods, at a distance (Osborn and Jaffé, 1998). This study thus represents a first detailed report of the ultrastructure of gland cells in spines which release these kinds of defensive volatiles.

4.2. The sack cells

In the neck gland in A. hylonomo the nuclei are large and irregularly shaped, similar to those found in the glands associated with the spines, and the cytoplasm contains rough endoplasmic reticulum. In the sacks, the many folds close to the basal membrane probably function to increase the absorption of substances. The high number of mitochondria in this area
suggested that large quantities of energy are utilised, indicating an active transport of substances towards the gland. It is probable then, that the cells which make up the sacks absorb defensive substances, or precursors for their production. The compounds are then orientated by the many microtubules towards the central reservoir where they are stored until required. The numerous microvilli that surround the reservoir permit a high rate of transport from the cells to the reservoir, in the same way as in the spinal glands.

Percy and MacDonald (1979) described the neck gland of Schizura concina (Notodontidae). The gross structure of the gland is different to that of Aphananthe hylonomes, having a posterior and an anterior gland, connected by an interglandular neck. Nevertheless, at a microscopic level, the cells of the posterior and anterior glands of S. concina and the sack cells of A. hylonomes are similar, the nuclei are irregularly shaped, and the cytoplasm contains numerous mitochondria and rough endoplasmic reticulum. These authors also report the presence of microtubules inside some of the cells, but consider that they have the function of maintaining the form of the cell. In A. hylonomes, we suggest that as well as this function, typical of the cytoskeleton, the microtubules may direct the transport of the compounds towards the reservoir, as in the axons of cells of the nervous system (Lusak, 1984). The presence of muscle fibre within the sacks indicates that these may contract when the larva is molested in order to expel the urticating substances. This contrasts with the continuous release of compounds by a gland responsible for the production of an aggregation pheromone in Rhyncophorous palmarum (Coleoptera: Cullionidae), reported by Sánchez et al. (1996) where no muscle fibre was observed.

4.3. The canal cells

The cells that surround the central canal of the neck gland are similar to the sack cells. The microvilli, next to the cuticle and the presence of mitochondria suggest that the cells actively secrete substances into the canal. This type of organisation corresponds to that of a type 1 cell (Noitrot and Quennedey, 1974; 1991), where the gland cell is in direct contact with the cuticle. The cuticle may allow the passage of the compounds through pores or canals, as is the case in the sternal gland of Trimerottermes geminatus (Termitidae: Nasutitermitinae) (Quennedey, 1972), or the secretion may diffuse through a continuous cuticle, as in the epithelial glands of social Hymenoptera (Bollen, 1987). In this study, no perforation was observed through the cuticle, which suggests that the volatiles pass through it by diffusion. The organisation of the canal cells is similar to that of the ellipsoid gland cells from the osmeteria of larvae of Papilio spp. (Lepidoptera: Papilionidae) (Crossley and Waterhouse, 1969; Lu and Chow, 1991). The osmeteria also contain a central canal, lined by cuticle and surrounded by cells with microvilli next to the cuticle. The osmeteria in Papilio spp. evert in the moment of defence in the same way as the neck gland in Aphananthe hylonomes, which leads us to suggest that in both cases, the structure of the cells is highly related to their function. Possibly, the presence of the cuticle ensures that the gland maintains its shape in the moment of eversion.

Thus the neck gland in A. hylonomes shares many of the structures present in the glands of other species of Lepidoptera which have been shown to be repellent to ants and other arthropods. Nonetheless this study represents a first report of these glands in the superfamly Papilionoidea.

In conclusion, the study of the ultrastructure of the spine and neck glands in A. hylonomes agrees with and is complementary to, biochemical studies which identify the presence of defensive compounds in both glands (Osborn and Jaffé, 1998). These investigations taken together thus suggest that they may function as sites for the synthesis and release of these compounds. Furthermore, the presence of specialised glands for the production of repellent substances for at least two ant species, Camponotus rufipes and Solenopsis geminata implies that these represent an important selective pressure in the evolution of this butterfly species.

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