Histochemical and Ultrastructural Analyses of the Epithelial Cells of the Body Surface Skin from the Terrestrial Slug, Incilaria fruhstorferi

Authors: Yamaguchi, Keiichiro, Seo, Naomi, and Furuta, Emiko

Source: Zoological Science, 17(8) : 1137-1146

Published By: Zoological Society of Japan

URL: https://doi.org/10.2108/zsj.17.1137

ZOOLOGICAL SCIENCE **17**: 1137–1146 (2000)

Histochemical and Ultrastructural Analyses of the Epithelial Cells of the Body Surface Skin from the Terrestrial Slug, *Incilaria fruhstorferi*

Keiichiro Yamaguchi[1], Naomi Seo[3] and Emiko Furuta[2]*

[1]*Institute for Medical Science and*
[2]*Department of Histology, Dokkyo University School of Medicine, Mibu, Tochigi 321-0293, and*
[3]*Department of Biology, Tokyo Medical University, Shinjuku-ku, Tokyo 160-8402, Japan*

ABSTRACT—Dorsal and ventral epithelium of the terrestrial slug, *Incilaria fruhstorferi*, is simple and consists of five cell types: microvillous, ciliated, round mucous, tubular mucous and channel. Microvillous cells were similar to human intestinal epithelial cells morphologically and functionally. At the base of microvilli, pinocytic vesicles which ultimately fused to form larger vacuoles, or multivesicular bodies were present. At the edge of tail or mouth, ciliated epithelial cells possessed the typical axonemes (9 plus 2 arrangement of microtubles). Mucous secretory cells were either tubular or round and their granules were membrane-bound and secreted by exocytosis. Granules of round mucous cells were proteinaceous but those of tubular cells were acidic mucopolysaccharides. Channel cells were elongate U-shaped and the central lumen was filled with a large amount of fluid (hemolymph). The function of channel cells is thought to remove hemolymph accumulated during hyperhydration. Our experiments of some markers-injection revealed that the fluid containing large molecules passed transcellularly from the hemolymph, across the basal or side region of the cell and into the central lumen. These results suggest that channel cell of the slug skin and vertebrate nephron showed some parallels in structure and function.

INTRODUCTION

Although the body surface of the terrestrial slug is covered with only simple columnar epithelial cells, the surface is prevented from evaporation and is protected from mechanical injuries. The reason is that the surface skin of the slug is covered with a large amount of mucus which is a characteristic secretion. Moreover, in spite of this fragile structure and moist conditions of cutaneous tissue, fairly resistant to invading microorganisms.

Many molluscs secrete a variety of substances which may have protective function such as skin pigments, lectins, opsonins, lysin *etc.* (Fountain and Campbell, 1984; Fountain, 1985; Iguchi *et al.*, 1982, 1985; Kubota *et al.*, 1985; Tripp, 1992; Kisugi *et al.*, 1992a, b; Furuta *et al.*, 1995). According to Furuta *et al.* (1995), the water soluble fraction of body surface mucus from *Incilaria fruhstorferi* plays an important role in the slug's immunodefense system. This fraction exerts opsonic and agglutinating activity for non-self materials, recognizing *N*-acetyl galactosamine (GalNAc). Moreover, Simkiss and Wilbur (1977) found that a range of large molecules such as beef hemoglobin (MW 68 Kd) passed out from the hemocoel onto the surface of the skin when it was injected into *Helix aspersa*. And we often observed that soon after injecting carbon particles into slugs, the particles appeared onto the skin's surface. According to Deyrup-Olsen and Martin (1982), the slug, *Ariolimax columbianus* could also exude large quantities of blood when stimulated physically, electrically, or by the intravascular injection of hypotonic fluids.

In order to elucidate internal defense system of molluscs, however, it is necessary to establish the structure of the terrestrial molluscan epidermis which plays a role as a barrier facilitating internal homeostasis and preventing the entry of pathogens from the environment into the body. The present paper provides histochemical and ultrastructural descriptions of the epidermal cells in the skin of the land slug, *I. fruhstorferi*.

MATERIALS AND METHODS

Slugs:

The largest slug in Japan, *I. fruhstorferi*, weighing 4.5–10 g ±1.0 g was maintained in the laboratory at room temperature for at least a month before use and fed on sweet potato, lettuce or mushroom.

* Corresponding author: Tel. +81-282-87-2124;
FAX. +81-282-86-1463.
E-mail: yamakei@dokkyomed.ac.jp

Injection of several markers:

To estimate the capacity of surface epithelium to transfer particles (carbon, fluosphare, yeast) or large molecules (FITC-dextran, Keyhole Limpet hemocyanin) from the hemocoelic space, these materials were injected into the hemocoel of slugs. They served as markers for particle or molecule sieving in fluorescence or electron micrographs.

The carbon (Pelican ink) and yeast particles were washed 5 times with 10 volumes of PBS (pH 7.4). After washing, they were diluted with same buffer. The fluosphares (red latex beads, 0.03 and 5.0 μm in diameter, Molecular Probes. Inc. USA) were directly diluted with 20 mM PBS (pH 7.4) and the FITC-dextran (MW 70 Kd, Molecular Probe. Inc. USA) and hemocyanin (BGN Co. Ltd. USA) were diluted with the same buffer.

Each solution or suspension was administered to each slug by injection into the hemocoel of 400 μg/g body weight in 20 mM PBS (pH 7.4). Each slug was then kept in a glass dish, for 3, 20 and 24 hr at room temperature.

Immunohistochemistry using anti-hemocyanin monoclonal antibody.

After excising the dorsal and ventral skins, samples were fixed in phosphate-buffered 10% formalin for 4 days, then dehydrated in a graded series of ethanols, embedded in paraffin, sectioned at 5 μm and mounted on precleaned glass slides. The following procedures were performed at room temperature.

Slides were deparaffinized and redehydrated. For immunohistochemical detection of hemocyanin pathway in the slug skin, specimens were treated with 3% H_2O_2 in distilled water (DW) for 15 min, then 2N HCl for 2 hr, and were then soaked in 20 mM phosphate buffer (pH 7.4) containing 110 mM NaCl (PBS). After treatment with 10% normal rabbit serum in PBS for 10 min, they were then incubated with primary antibodies, i.e. goat anti-hemocyanin (from Keyhole Limpet) monoclonal antibody (BGN Co. Ltd. USA, dilution of 1:200) in 50 mM tris buffer (pH 7.2) for 60 min. After rinsing with PBS, they were treated with biotinylated rabbit anti-goat IgG (Nichirei kit, Nichirei Co. Ltd. Tokyo, Japan) for 30 min. They were then rinsed with PBS and treated with streptavidin-horse radish peroxidase conjugates (Nichirei kit) for 30 min. After rinsing with PBS, sections were immersed for 10 min in diaminobenzidine containing H_2O_2 (Nichirei kit). As a control experiment, sections were treated with normal rabbit serum instead of anti-hemocyanin monoclonal antibody. No control sections showed any signal.

Histochemistry:

Several histochemical procedures were performed to reveal carbohydrates. Acidic mucosubstances were demonstrated by alcian blue (pH 2.5)(Bonet and Huguet, 1985) or by toluidine blue, which stains the acidic mucus metachromatically (Gabe, 1968). Moreover, we examined the staining pattern of the epidermis (dorsal and ventral sides) after incubation with seven lectins. The following procedures were performed at room temperature. After deparaffinization, specimens

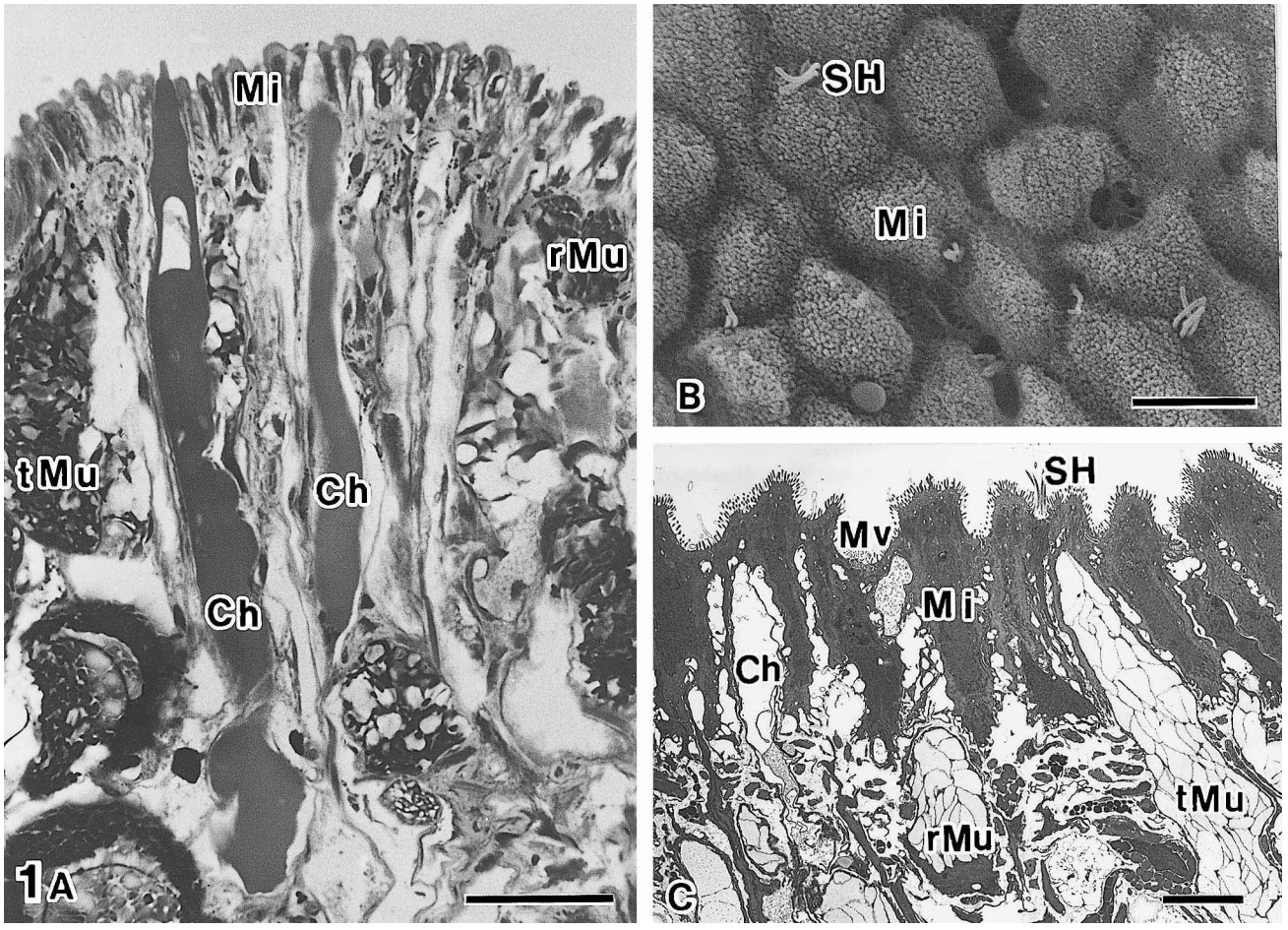

Fig. 1. Five cell types in the epidermis of dorsal skin from terrestrial slugs, *Incilaria fruhstorferi*. The epidermis is composed of 5 cell types: surface microvillous (Mi), ciliated (see Fig. 4), round mucous (rMu), tubular mucous (tMu) and channel (Ch). A; Light microphotograph of semithin section stained with toluidine blue. scale bar =50 μm. B; Scanning electron microscopic (SEM) photograph. scale bar =5 μm. SH, sensory hair C; Transmission electron microscopic (TEM) photograph. scale bar =5 μm.

were treated with 0.3% H_2O_2 in DW for 15 min to block the endogenous peroxidase activity and rinsed with PBS (pH 7.4). They were treated with 0.1% normal bovine serum albumin (BSA) in 0.1 M Hepes buffer (HB, pH 7.5) for 30 min and rinsed again with HB. Sections were incubated with one of the following seven biotinylated lectins (25 μg/ml) in 0.1% BSA-HB for 30 min, purchased from Vector Laboratories, USA: concanavalin A (Con A), *Dolishos biflorus* agglutinin (DBA), peanut agglutinin (PNA), *Ricinus communis* agglutinin (RCA), soybean agglutinin (SBA), *Ulex europeus* agglutinin (UEA-1) and wheat germ agglutinin (WGA). After incubation with lectin, the sections were rinsed with HB and then incubated with avidin-biotin-peroxidase complex (ABC kit, Vector) in 0.1% BSA-HB for 30 min. After rinsing with HB, the sections were reacted with a mixture of 0.05% 3,3'-diaminobenzidine-4HCl (Wako, Tokyo, Japan) and 0.01% H_2O_2 in 0.05M Tris buffer (pH 7.2) for 5 min. They were rinsed with DW, dehydrated and mounted with Entellan New (Merck, Darmstadt, Germany). The staining intensities were scored as - negative staining, ± weak staining and + positive staining. As a control procedure, to confirm the specificity of the histochemical reactions, several sections were preincubated with appropriate hapten sugars and then incubated with biotinylated lectins in the presence of hapten sugars. Sugars used were; 0.2 M α-metyl-*D*-mannosid (Sigma, St. Louis, USA) for Con A, 0.2 M *N*-acetyl-*D*-galactosamine (Aldrich, Wisconsin, USA) for DAB and SBA, 0.1 M *D*-galactose (Wako) for PNA, 0.2 M lactose (Wako) for RCA-1, 0.1 M *L*-fucose (Aldrich) for UEA-1, and 0.2M *N*-acetyl-*D*-glucosamine (Wako) for WGA. Nuclei were counterstained with hematoxylin

Electron microscopy:

For transmission electron microscopy, the skin from dorsal or ventral surface was fixed for 1.5 hr in 1.6% paraformaldehyde - 3% glutaraldehyde in 0.1M cacodylate buffer (pH 7.4). Fixed tissues were rinsed with the same buffer (pH 7.4), postfixed with 1% O_sO_4 for 1.5 hr, dehydrated, and embedded in Epon 812 resin. Ultrathin sections were obtained with a Super Nova ultramicrotome (Reichert-Jung, Austria), double stained with uranyl acetate and lead citrate, and examined in a JEM-1210 electron microscope (JEOL, Tokyo Japan). Semithin sections, approximately 1–2 μm thick, stained with an alkaline solution of toluidine blue, were also stained for light microscopy. For scanning electron microscopy, the double fixed tissues were dehydrated, critical point dried, sputter-coated with Au-Pd and examined with a S-650 scanning electron microscope (Hitachi, Tokyo Japan).

RESULTS

Epidermal cells of the land slug, *I. fruhstorferi*, depend-

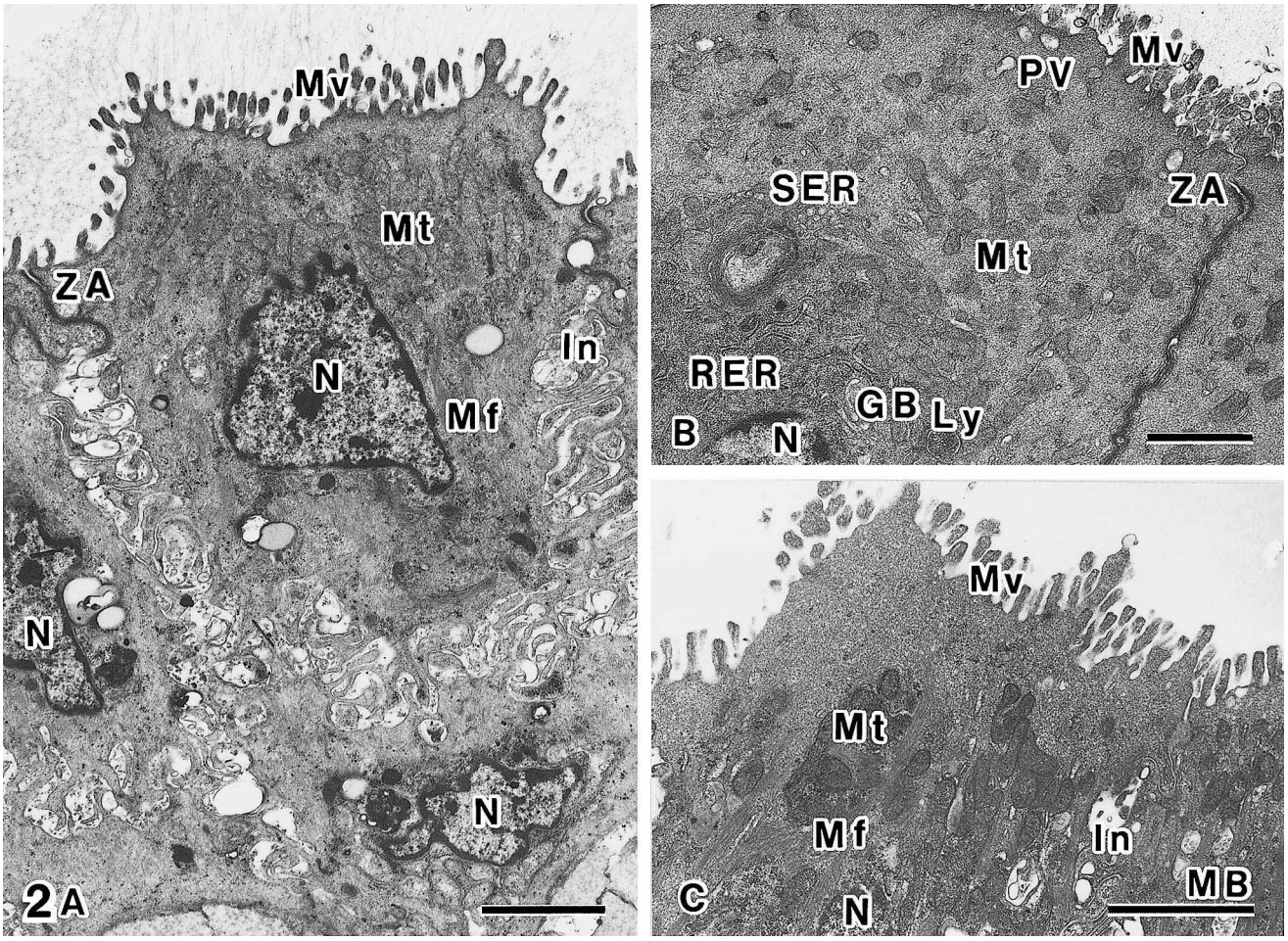

Fig. 2. TEM micrographs of microvillous cells of the epidermis from slug's skin. A; Microvillous cells of dorsal skin, B; Microvillous cells of ventral skin, C; Apical region of microvillous cells of dorsal skin. Microvillous cells connected each other with interdigitation (In) and clung to *zonula adherens* (ZA). These cells contained many mitochondria (Mt) which were oval or oblong in shape and many microfilaments (Mf). These components were arranged parallel to the long axis of cell. Cells often possessed smooth endoplasmic reticulum (SER), rough endoplasmic reticulum (RER), pinocytic vesicles (PV), lysosomes (Ly) and multivesicular bodies (MB). N, nucleus; GB, Golgi body; Mv, microvilli. Scale bars =2 μm.

ing on the region of the body, vary from cuboidal to columnar in form. The cells between dorsal and ventral skin, *i.e.* at the edge of tail or mouth, are cuboidal. The external surface of the epidermis was composed of a simple microvillous cell layer which held in place the covering of mucus. The cell layer is supported by a mat of connective tissue through which muscle fibers ran. As shown in Fig. 1, five main types of cells were distinguished: (1) microvillous, (2) ciliated (see Fig. 4), (3) round mucous, (4) tubular mucous and (5) channel.

(1) *Microvillous cells*: These cells ranged from 14–21 μm in height (Figs. 1C, 2) and occasionally protrusions were observed at the tips of the microvilli. The epithelia were confused with the numerous intercellular spaces which were very large and often extended (Fig. 2). The epidermal cells closely clung to each other by *zonula adherens* (175–225 Å) in the apical region of cells. The *zonula adherens* is the most prominent in cells with a well-developed brush border. Below the microvilli of the border, the apical cytoplasm is traversed at the level of the *zonula adherens* by a mat of interwoven filaments the so-called terminal web (Fawcett, 1981). The 17 to 18 nm space between parallel membranes of adjacent cells was filled with continuous layer of dense materials. Below this *zonula* the membrane interdigitations were present (Fig. 2). The microvillous epidermal cells often possessed fine filaments (actin filaments)(Fig. 2C) which ran toward the tip of the microvilli, these filaments arose from the "terminal web" that was devoid of cell organelles. The terminal web was found besides several fibers and vesicles, these components were similar to core filaments of brush border associated with human intestinal epithelia. Irregularly shaped mitochondria were gathered chiefly in the cell's apical region (Fig. 2). The mitochondria usually had an oval to slightly oblong form; the long axis ran parallel to the length of the cell (Fig. 2). Dense bodies, multivesicular bodies (MB) and several large and small vesicles were interspersed among the mitochondria. The dense bodies which were considered as lysosomes were about 0.5–1.0 μm in diameter. The multivesicular bodies usually contained small vesicles of similar size. Golgi bodies were rarely observed in microvillous cell (Fig. 2B). The endoplasmic reticulum was mainly smooth endoplasmic reticulum (SER), and numerous free ribosomes were observed throughout the cytoplasm (Fig. 2B).

Microfilaments frequently occurred in bundles, especially along the sides of the nucleus and usually ran parallel with the cell axis (Fig. 2C). They extended from the interior of the cell into microvilli. At the base of microvilli, pinocytic vesicles

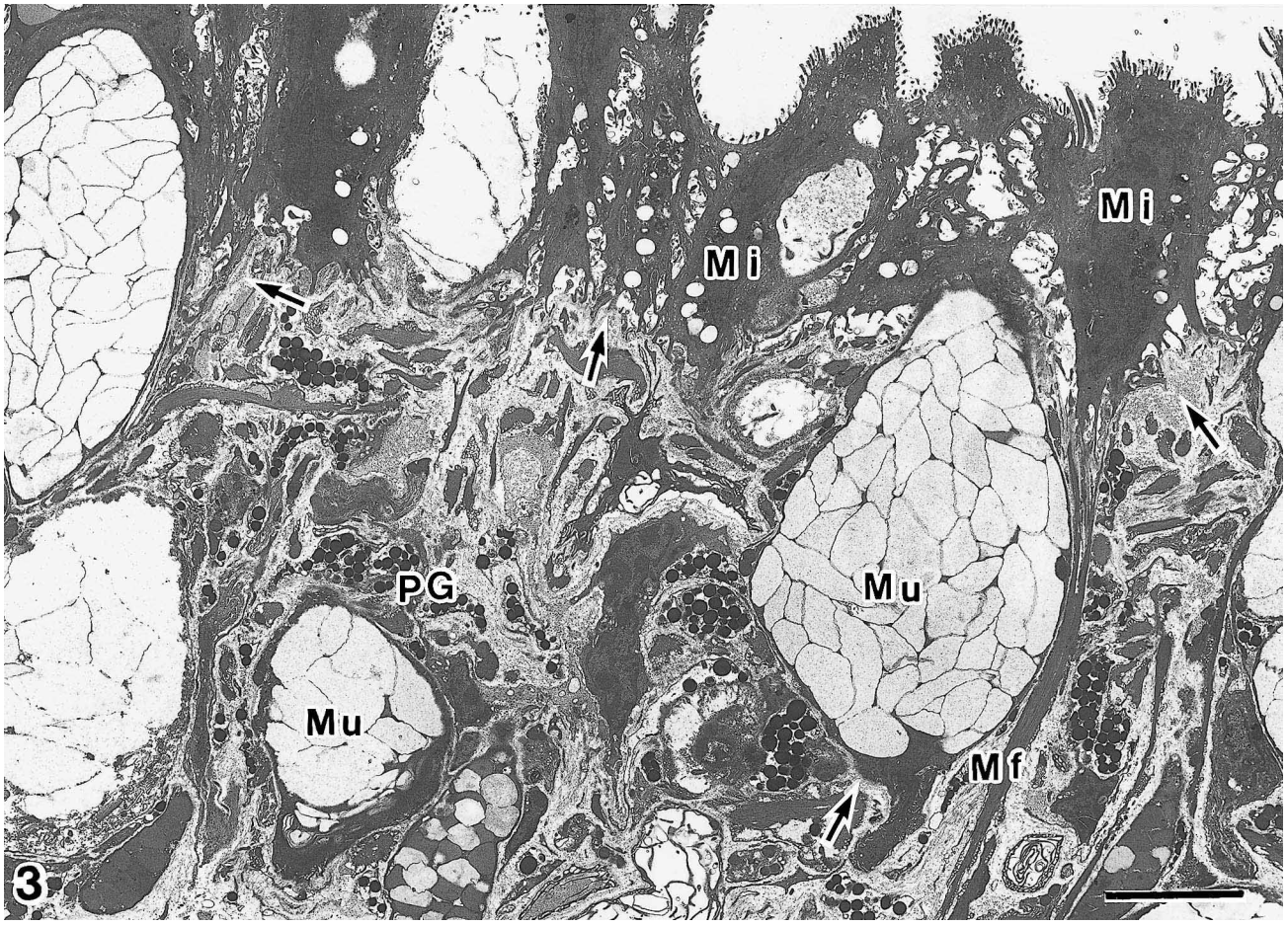

Fig. 3. TEM micrograph of basement membrane of epidermal cell layer. In the dorsal skin, basement membrane (arrows) ran indentedly underneath the microvillous cells (Mi) and mucous cells (Mu), and was broad at places . PG, pigment granule; Mf, muscle fiber. Scale bar =5 μm.

(PV) and some multivesicular bodies (MB) which contained small vesicles were often observed (Fig. 2). The basement membrane appeared to have occasional indentations or thrown into basal folds, and at places broad (Fig. 3).

(2) *Ciliated cells*: At the edge of tail or mouth, epidermal cells were ciliated. The cilia possessed very long striated roots, up to 2–9 μm in length and the typical axonemes (9 plus 2 arrangement of microtubles) (Fig. 4). Cell connection of ciliated cells was similar to that of microvillous cells. The cytoplasm was closely packed with many mitochondria and interspersed with glycogen-like deposits.

The presence of mucous glands is well established in molluscs, and the cells typically possess a cell body located in the subepidermal connective tissue and a secretory process extending to the surface of the epidermis.

(3) *Round mucous cells*: The round mucous cells were more abundant on the ventral surface than on the dorsal surface (Table 1). They were located directly underneath the epidermal cells and opened onto the surface with the short neck. In our regenerating experiments, the cell body moves more deeply into the dermis as the neck grows (Yamaguchi *et al*., 1999). The cell was globose, measuring about 10–16 μm in diameter (Fig. 5A) and its neck was anchored to adjacent columnar epithelial cells by means of a junctional complex (Fig. 1C). The oval nucleus located at the center of the cell, measuring about 3.6–5.8 μm in diameter. Membrane-bound secretory granules were present around the nucleus and the cell body became progressively distended with secretory granules becoming more prominent. Chromatins showed heterochromatic granules and a nucleolus was present. Golgi bodies were stacked and consisted of seven to nine lamellae (Fig. 5B). The cytoplasm was rich in rough endoplasmic reticulum (RER). Few mitochondria laid among rich RER. Basement membrane which was found outside the body and the neck of the round mucous cells ran the adjoining columnar epithelial cells. Fine muscle fibers were also observed around the cell body.

(4) *Tubular mucous cells*: Tubular cells possessed a cell body located in the musculature and a long secretory process extending to the surface of the epidermis (Fig. 5C). The nucleus laid at the base of the cell where Golgi bodies and RER existed along with most of the cytoplasm. And the cell possessed a distal region which extended into the long secretory process packed with membrane-bound mucous products. These mucous granules were secreted by exocytosis (Fig. 5C). Golgi bodies were prominent and consisted of seven to nine lamellae. The cytoplasm contained numerous free ribosomes.

(5) *Channel cells*: The channel cell was the large cell which was lightly stained and usually homogeneous microscopically (Fig. 6). The nucleus was also large and laid in a thin mass of cytoplasm at the basal portion of the cell. The cytoplasmic envelop was very thin except in the region of the nucleus, which contained RER, a few mitochondria, many free ribosomes, Golgi bodies and various pinocytic vesicle-like vesicles (Fig. 6B).

As illustrated in Fig. 7, the basement membrane sur-

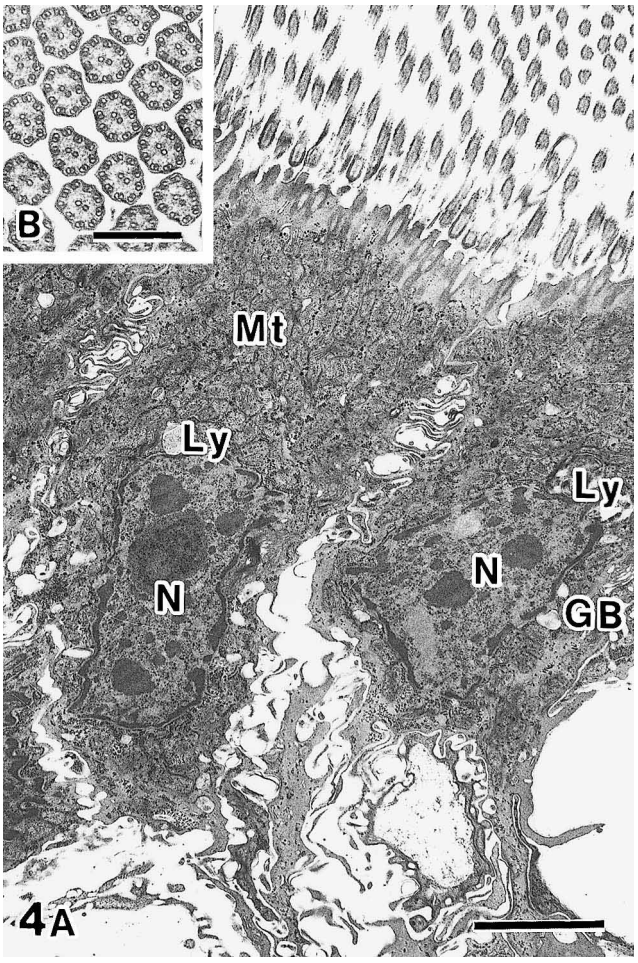

Fig. 4. TEM micrograph of ciliated cells from the edge of dorsal and ventral skin of slug. Ciliated cells possessed many cilia with typical axonemes (9 plus 2 complex) that were 2–9 μm in length. The cell connection was similar to that of microvillous cells. The cytoplasm contained many mitochondria (Mt) in the apical region and the nucleus (N) laid at the base of the cell. Ly; lysosome, GB; Golgi body. A: ciliated cells, scale bar=2 μm, B: axonemes, scale bar=0.5 μm.

Table 1. Distribution and differences of histological observations of skin mucous cells from land slug, *Incilaria fruhstorferi*. Detection of mucous mucopolysaccarides was performed by the reaction of 7 kinds of biotinylated lectins.

	Dorsal skin		Ventral skin	
	Round cell	Tubular cell	Round cell	Tubular cell
MT stain	not many	numerous	numerous	not many
AB	–	+	–	+
Con A	–	–	–	–
WGA	+	–	+	–
RCA	–	+	–	+
DBA	–	–	–	–
SBA	±	–	±	–
PNA	–	–	–	–
UEA	–	–	–	–

Reactions; +: positive ±: weekly positive –: negative
MT stain: Masson trichrome staining AB: alcian blue Con A: concanavalin A WGA: wheat germ agglutinin RCA: *Ricinus communis* agglutinin DBA: *Dolishos biflorus* agglutinin SBA: soybean agglutinin PNA: peanut agglutinin UEA: *Ulex europeus* agglutinin

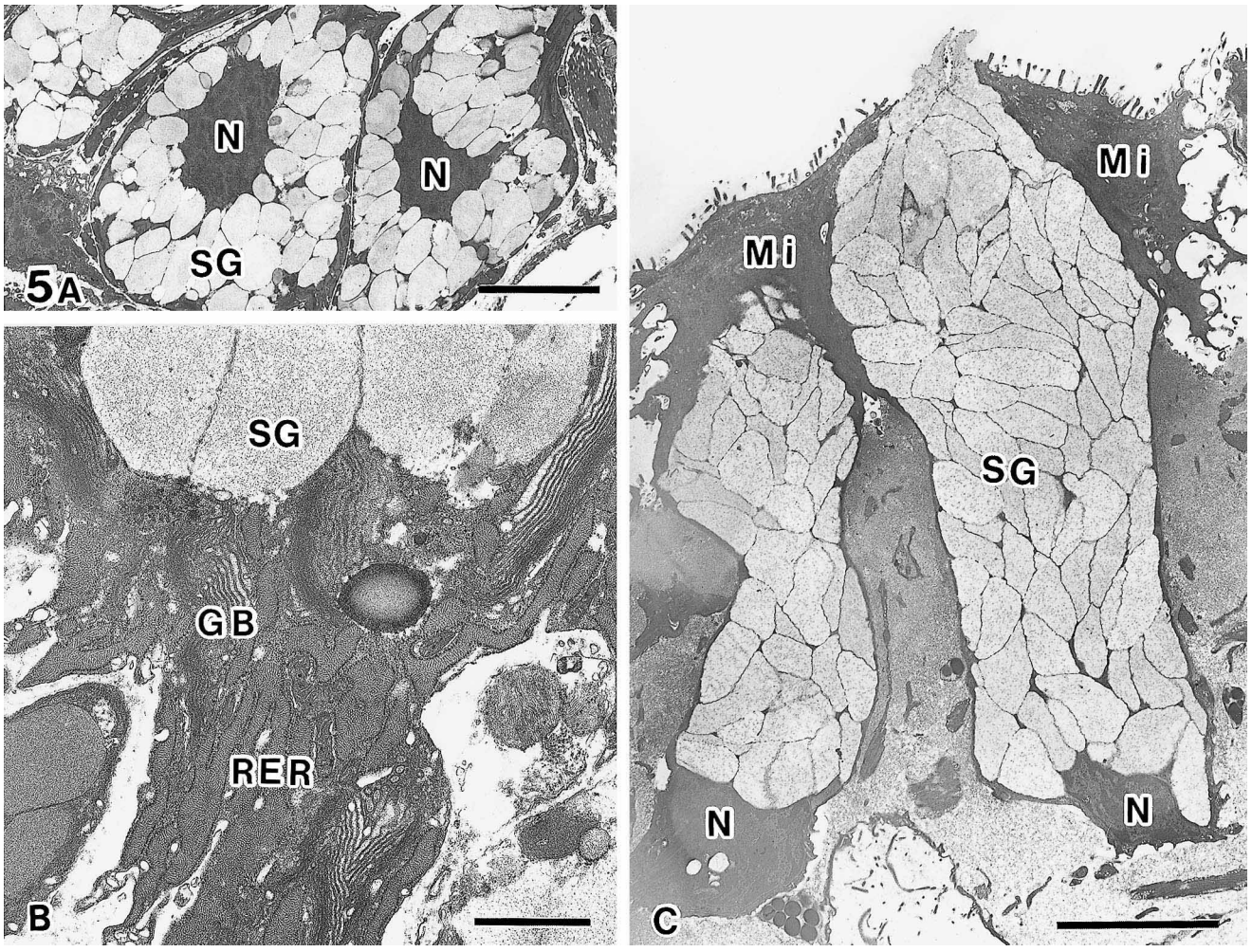

Fig. 5. TEM micrographs of two types of mucous cells from the ventral and dorsal slug's skin. A, B: round mucous cell from the ventral skin; C: tubular mucous cell from dorsal skin. The round mucous cells were abundantly observed on the ventral skin rather than on the dorsal skin. Cell bodies located directly underneath the epidermal cells and opened onto the surface with the short necks. The cell mainly contained many secretory granules (SG) around a oval nucleus (N) which located in the center and stacked well-developed Golgi bodies (GB) and rough endoplasmic reticulum (RER). On the other hand, tubular mucous cells were observed in the dorsal skin rather than in the ventral skin. This cell possessed a cell body located in the musculature and a long process which were filled with many mucous granules and its nucleus laid at the base. Golgi bodies existed along with the nucleus. Mucous granules were secreted by exocytosis. Mi, microvillous cell. Scale bars; A =5 μm, B=1 μm, C=5 μm.

rounded the basal and lateral side of the cell, and very thin smooth muscles and pigment cells also surrounded the cell. The shape of the cell was cylindrical, and long U-shaped cytoplasm.

Histochemical characteristics of three secretory cells of the epidermis.

(1) *Round mucous cells*: Granules in these cells were negative for acidic mucopolysaccharide and slightly positive for proteins. Lectin immunohistochemistry showed that WGA positively (Fig. 8) and SBA slightly stained the granules of round mucous cell (Table 1).

(2) *Tubular mucous cells*: The staining reaction of the secretion was strongly positive with alcian blue (pH 2.5) showing the granules of acidic mucopolysaccharide and toluidine blue staining as metachromatic. Lectin immunochemistry showed that only *Ricinus communis* agglutinin (RCA) slightly stained the granules of the tubular mucous cells (Table 1, Fig. 8).

(3) *Channel cells*: Three hours after injection of carbon particles, fluoshares (red, 0.03 μm in diameter), or FITC-dextran (MW 70Kd), these cell markers were through out the body wall (Table 2). Fig. 6C shows the basal region of the channel cell which took up the particles by pinocytosis and then released to the lumen of U-shaped cells by exocytosis. At this time, the structure of junctional complexes did not show any changes. The staining reaction of anti-hemocyanin monoclonal antibody was positive in the lumen (Fig. 9).

DISCUSSION

The epidermis of terrestrial slugs function not only as an integument, but it also plays a role in osmoregulation, respiration, locomotion and host defense (Furuta *et al.*, 1995; Yuasa *et al.*,1998) where is covered with secreted mucus from mu-

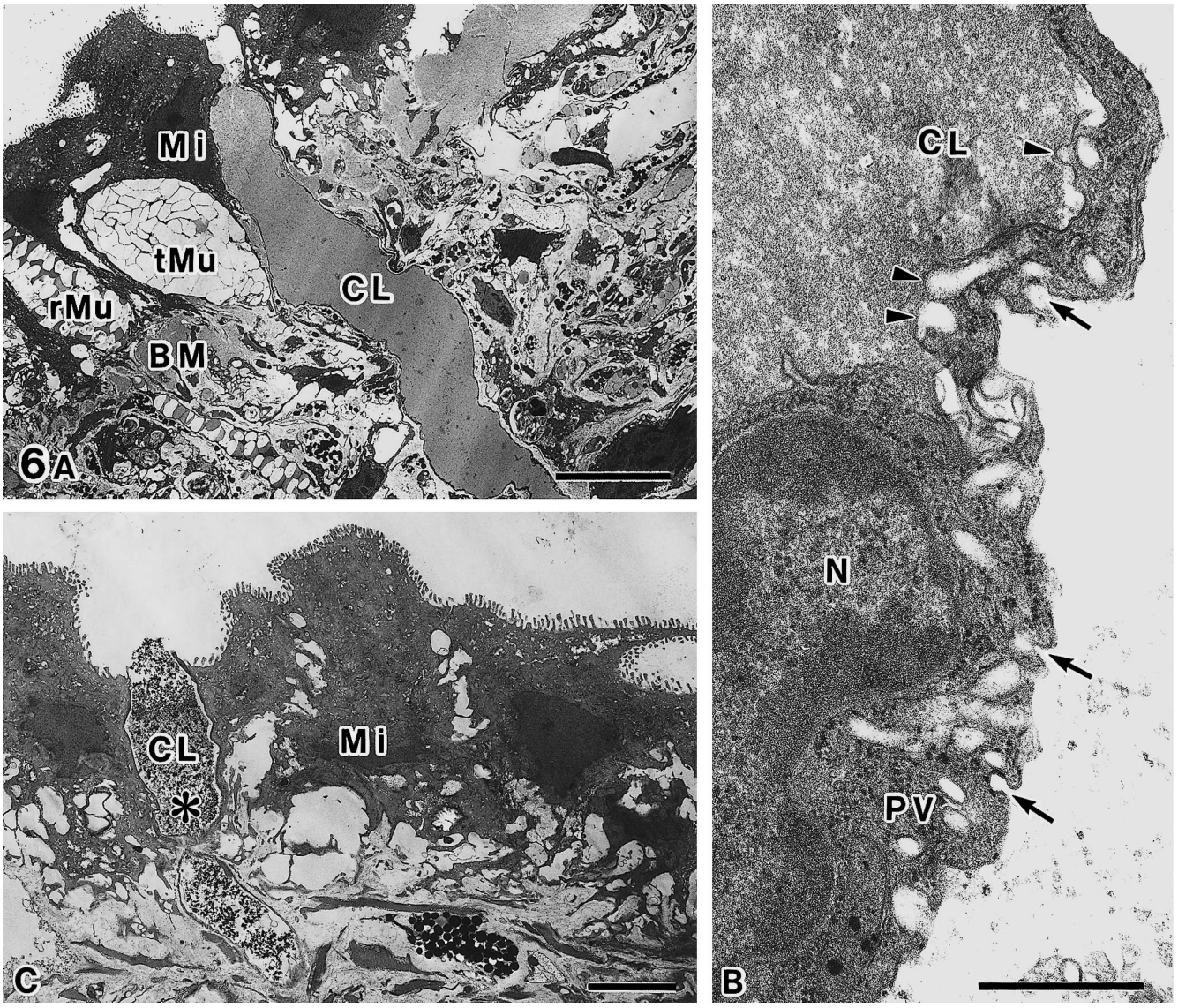

Fig. 6. TEM micrographs of channel cells (Ch) from the slug's skin. A, a channel cell elongated from the surface of epidermis to the connective tissue; B, pinocytic and exocytic structural components of channel cell; C, pouring carbon particles (*) through the body wall of channel cell 3 hr after injection. This cell was long U-shaped and its nucleus (N) was laid in a thin mass of cytoplasm at the basal portion. Various invaginations of cell membrane reflected endocytosis (arrows) or exocytosis (arrowheads). Mi, microvillous cell; tMu, tubular mucous cell: rMu, round mucous cell; CL, central lumen of channel cell; BM, basement membrane; PV, pinocytic vesicle. Scale bars, A=10 μm; B=0.5 μm; C=5 μm

cous secretory cells. The external surface of the epidermis was composed of microvillous layer which increased the dorsal surface area, and most of the exposed surface consisted of microvillous-bearing epidermal cells and the mucus producing glands which consisted of simple goblet cells forming a secretion that stained for either protein or acid polysaccharide components. The epidermal cells possessed microfilaments (Fig. 3) which might function as a cytoskeleton and a contractive structure of the cells. The slug body usually could be stretched until its length is increased about 2–3 times. In *Lymnaea stagnalis*, Zylstra (1972) reported that the epidermal cells lining the mouth -they were submitted to a great deal of wear in the eating process- contained numerous microfilaments, and that the microfilaments appeared to function as a cytoskeleton providing a more firm matrix to resist wear by giving strength to the cells.

In the leech which possesses mucus-rich skin, two types of mucous cells are found between epidermal cells (Molinas and Huguet, 1993) and one type (more common type in the leech skin) is involved in mucus formation by their secretion of mucosubstances slightly positive for carboxylated mucopolysaccharides and for proteins. The other cell type occurs in small numbers and supplies a carboxylated mucopolysaccharide component together with neutral and sulfated fractions. However, in molluscs many investigators reported various types of mucous gland cells, for example, in *L. stagnalis*; Zylstra (1972a, b) described 13 different types of mucous cells, in *H. aspersa*: 8 types of unicellular glands (Campion, 1961), and in *Pattela vulgata*: 9 types of glands on the foot alone (Grenon and Walker 1978).

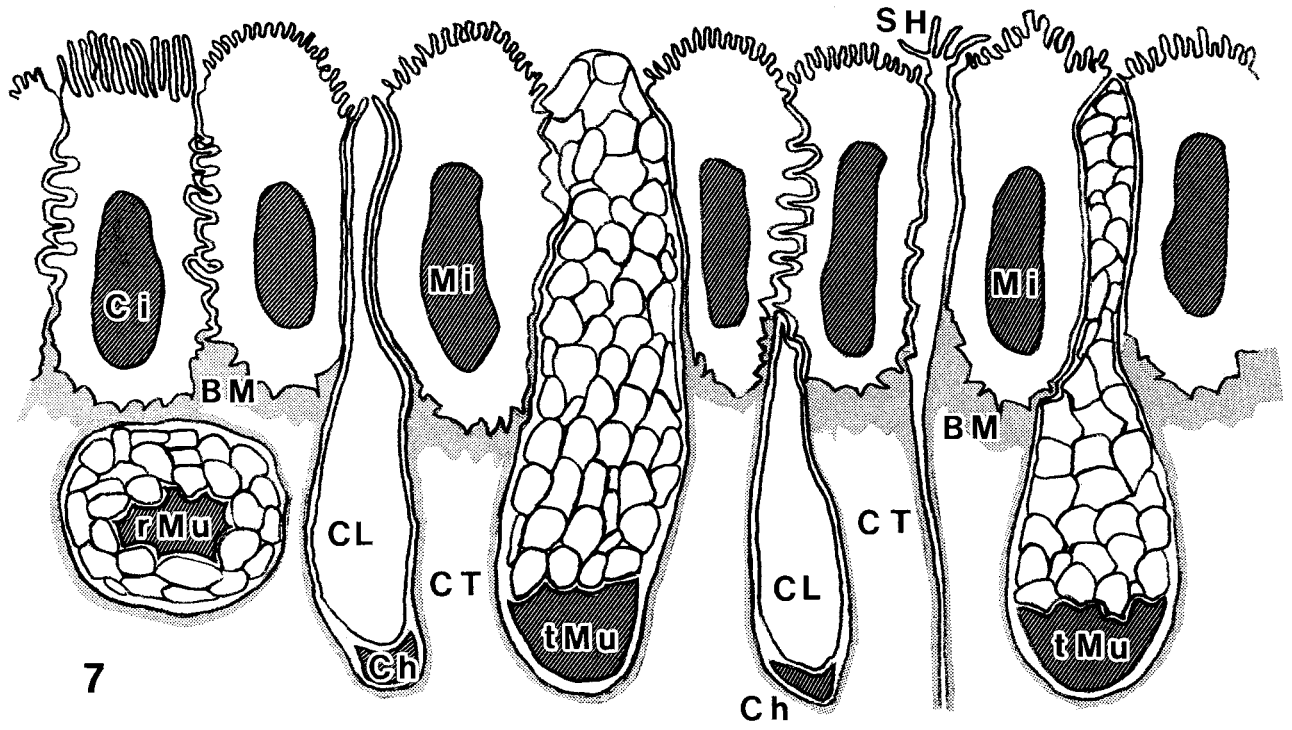

Fig. 7. Diagram showing the ultrastructural feature of epidermis of slug skin. The epidermis of slug skin is mainly composed of 5 cell types: microvillous (Mi), ciliated (Ci), round mucous (rMu), tubular mucous (tMu) and channel (Ch). The cells typically possess a cell body located in the subepidermal connective tissue (CT) and a secretory process extending to the surface of the epidermis. Basement membrane (BM) of these 5 cell types abuts upon the edge of connective tissue. CL, central lumen of channel cell; SH, sensory hair.

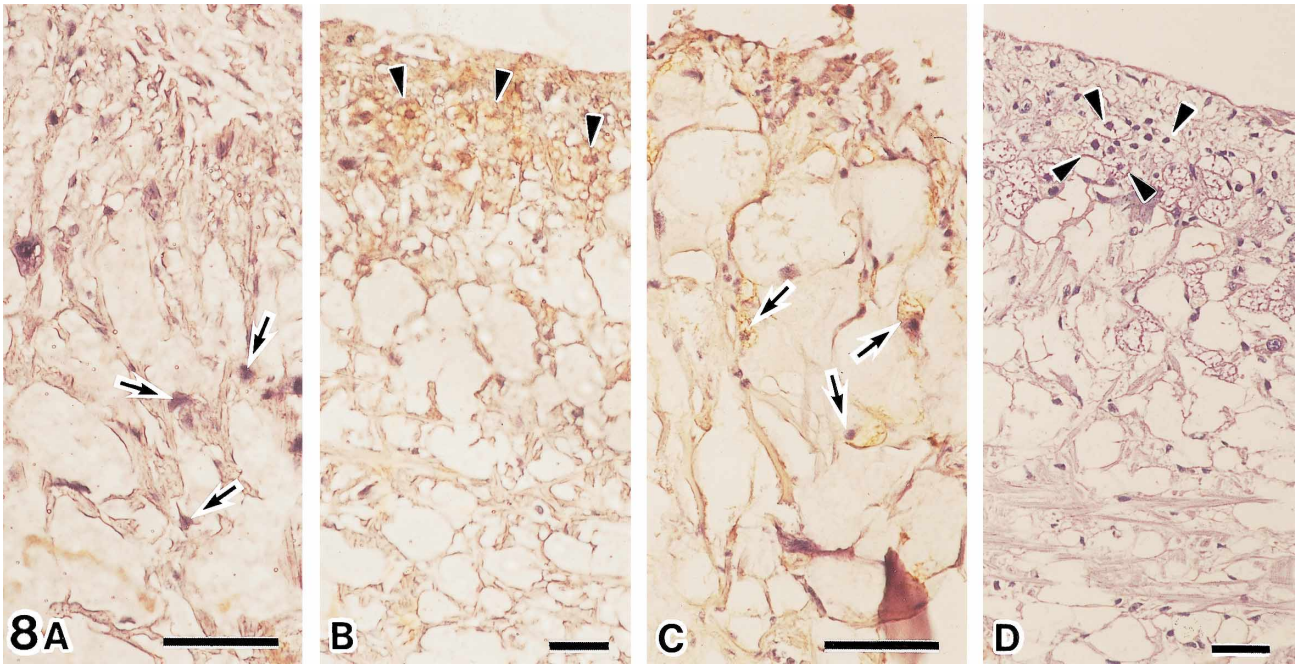

Fig. 8. Detection of lectins (WGA, RCA) in the dorsal and ventral slug's skins. Deparaffinized and dehydrated sections were incubated with biotinylated WGA or RCA, reacted with avidin-biotin-peroxidase and stained with diaminobenzidine (ABC method). A; WGA staining of tubular mucous cells (arrows) of dorsal skin, B; WGA staining of round mucous cells (arrowheads) of ventral skin, C; RCA staining of dorsal mucous cells, D; RCA staining of ventral mucous cells. Tubular mucous cells of dorsal skin were positively stained with RCA but not with WGA. On the other hand, round mucous cells of ventral skin were slightly stained with WGA but not RCA. Scale bars =10 μm

Table 2. Estimation of the capacity of surface epithelium to transfer large molecules and particles from the hemocoelic space.

Materials	Size or MW	Capacity of passing
Sheep red blood cell	5 μm	No
Yeast particle	1 μm	No
Latex bead (a)	5 μm	No
(b)	30 nm	Yes
Carbon particle	30 nm	Yes
FITC-dextran	70 Kd	Yes
Keyhole Limpet hemocyanin	400 Kd	Yes

Each material was injected into slug's hemocoel of 400 μg/g body weight in 20mM PBS (pH 7.4) and estimated the capacity of surface epithelium to transfer large molecules and particles 3 hr after injection.

We reported here the existence of two major types of secretory cells. These two cells were similar to the leech-skin's secretory cells, but were different from the secretory glands and/or cells of other molluscs. In a study on nudibranchia, *Onchidorus muricata*, Skidmore and Rivera (1982) concluded that "the confusing variety of secretory cell types were distributed over the gastropod body and foot". Functionally considered, the molluscan mucus contains lectins (Fountain, 1985; Fountain and Campbell, 1984; Iguchi *et al.*, 1985; Kubota *et al.*, 1985; Furuta *et al.*, 1995; Yuasa *et al.*, 1998) and antibacterial components (Iguchi *et al.*, 1982). The water soluble fraction of the slug mucus also inhibited growth of Gram positive bacteria and fungus (in preparing for publication). The microvillous epithelial cells possessed numerous large vesicles and microvesicles (Fig. 5). This phenomenon is often probable to show endocytotic activity. Indeed, Bevelander and Nakamura (1966) reported that the endocytosed materials by mantle epithelial cells were fused with lysosomes and were thereby digested. Especially, in terrestrial molluscs, *Agriolimax reticulatus*, endocytosis and the associated lysosomal activity occurred on the foot epithelium (Ryder and Bowen, 1977). Multivesicular bodies were also present approximately 0.4–0.5 μm in diameter. They were enclosed by single membrane and contained small vesicles (Fig. 5). We observed the most intriguing cells which existed among columnar epithelial cells. They were lightly stained and rather homogeneous in appearance. Under electron microscope the cell was elongate U-shaped and its central lumen was filled with fluid, and surrounded by a thin layer of cytoplasm and with a large basal nucleus. Moreover, it was additionally intriguing to observe

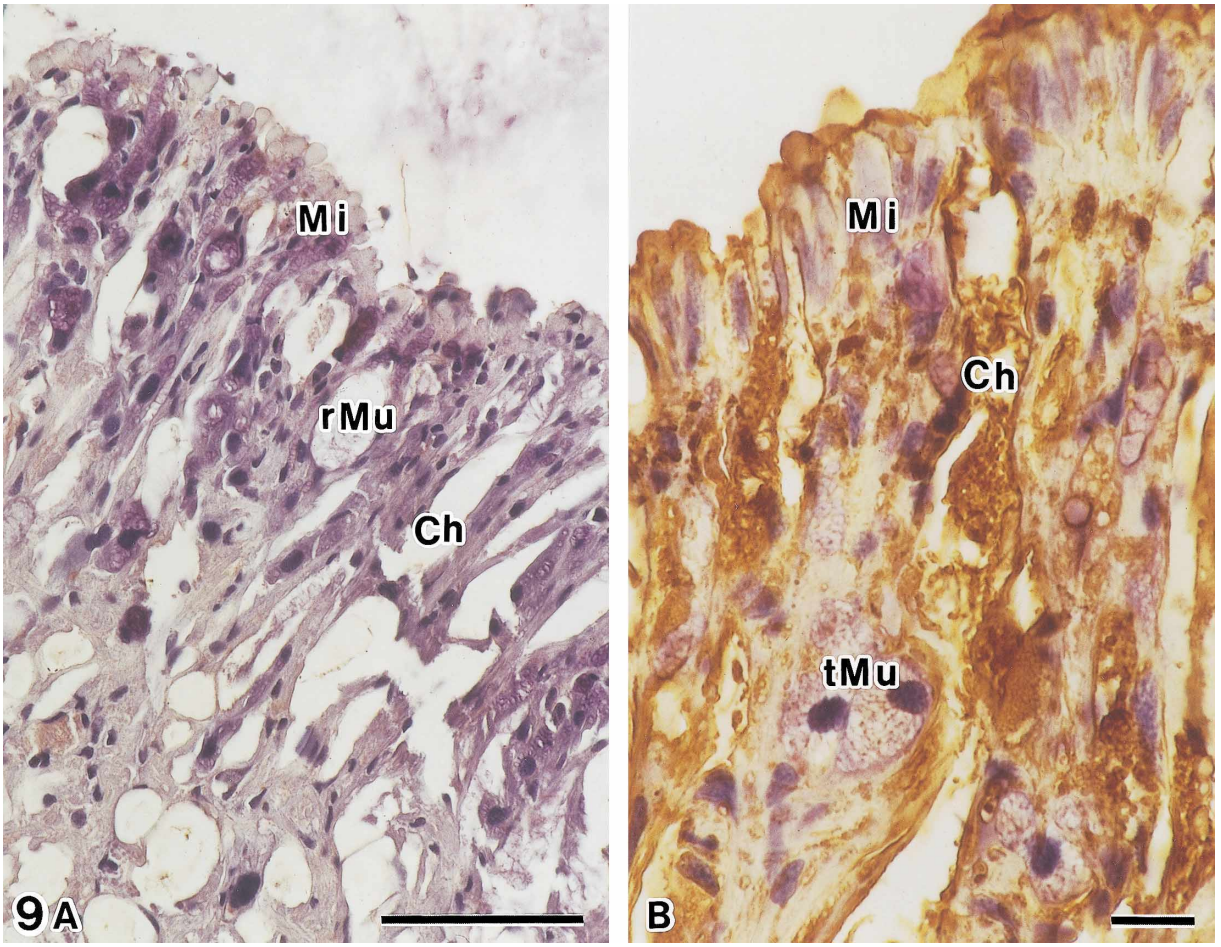

Fig. 9. Immunohistochemical detection of hemocyanin passway in the slug's skin. Slug hemocyanin was conjugated with goat anti-hemocyanin (from Keyhole Limpet) and the conjugate was detected by diaminobenzidine staining after streptavidin-horse radish peroxidase treatment. A, control; B, hemocyanin staining (1:200). Mi, microvillous cell; Ch, channel cell; tMu, tubular mucous cell; rMu, round mucous cell. Scale bars =10 μm

the traversal of very large molecules such as hemocyanin, dextran (MW 70Kd), carbon and latex beads (0.03 μm in diameter) 30 min after injection. Luchtel *et al*. (1984) described these cells as "channel cells" and suggested that fluid passed in transcellular tubules from the blood, across the cell's basal region, and into the central lumen from which the fluid might be passed onto the apical surface. Our observations supported the suggestion of Luchtel *et al*..

By this means the U-shaped cells (channel cells) are thought to remove (release) hemolymph accumulated during hyperhydration. Indeed, Martin and Deyrup-Olsen (1986) described the function of channel cells. Channel cells of slug's skin and the vertebrate nephron show some intriguing parallels in structure and function, and in each, a primary fluid was formed by ultrafiltration through an epithelial basement membrane (Fujita and Fujita, 1992). Moreover, they reported that slugs could exert neurohormonal control over its channel cells.

In recent years, it is said that immune system is closely associated with neuroendocrinological system, aptly termed neuroendocrinoimmunology. Further studies should be in progress to elucidate these relationships in the near future.

ACKNOWLEDGMENT

We thank Professor Edwin L. Cooper, Department of Neurobiology, UCLA Medical Center, for kind revision of the manuscript.

REFERENCES

Bevelander G, Nakamura H (1966) Correlation of lysosomal activity and ingestion by the mantle epithelium. Biol Bull 131:78–82

Bonet S, Huguet G (1985) Teqcniqes habituals de coloracions semifines de material incluit en glicol metacrilat. Sci Gerundensis 10: 23–32

Campion M (1961) The structure and function of the cutaneous glands in *Helix aspersa*. Q J Microsc Sci 102: 195–216

Deyrup-Olsen I, Martin AW (1982) Surface exudation in terrestrial slugs. Comp Biochem Physiol C72: 45–51

Fawcett DW (1981) The Cell. 2nd ed, WB Saunders Company, Philadelphia

Fountain DW, Campbell BA (1984) A lectin isolate from mucus of *H. aspersa*. Comp Biochem Physiol 77B: 419–425

Fountain DW (1985) The lectin-like activity of *Helix aspersa* mucus Comp Biol Physiol B795–800

Fujita H, Fujita T (1992) Textbook of Histology. part 2, Igakushoin Ltd., Tokyo, pp200–209 (in Japanese)

Furuta E, Takagi T, Yamaguchi K, Shimozawa A (1995) *Incilaria* mucus agglutinated human erythrocytes. J Exp Zool 271: 340–347

Grenon JF, Walker G (1978) The histology and histochemistry of the pedal glandular system of two limpets, *Patella vulgata* and *Acmaea tesutata* (Gastropoda: Prosobranchia). J Mar Biol Assoc U K 58: 803–816

Gabe AW (1968) Techniques histologiques. Paris Masson et Cie Iguchi SMM, Aikawa T, Matsumoto JJ (1982) Antibacterial activity of snail mucus mucin. Comp Biochem Physiol A72: 571–574

Iguchi SMM, Momori TM, Egawa K (1985) An N-acetylneuraminic acid-specific lectin from the body surface mucus of African giant snail. Comp Biochem Physiol B81: 897–900

Kisugi J, Kamiya H, Yamazaki M (1992a) Biopolymers from marine invertebrates. XII A novel cytolytic factor from a hermit crab, *Clibanarius longiarsis*. Chem Pharm Bull 40: 1641–1643

Kisugi J, Ohye H, kamiya H, Yamazaki M (1992b) Biopolymers from marine invertebrates. XIII Characterization of an antibacterial protein Dolabellanin A, from the albumin gland of sea hare, *Dolabella auriculata*. Chem Pharm Bull 40: 1537–1539

Kubota Y, Watanabe Y, Otsuka H, Tamiya T, Tsuchiya T, Matsumoto JJ (1985) Purification and characterization of an antibacterial factor from snail mucus. Comp Biochem Physiol C82: 345–348

Luchtel DL, Martin AW, Deyrup-Olsen I (1984) The channel cell of the terrestrial slug *Ariolimax columbians* (Stylommatophora: Arionidae) Cell Tissue Res 235: 145–151

Martin AW, Deyrup-Olsen I (1986) Function of the epidermal channel cells of the body wall of a terrestrial slug, *Ariolimax columbians* . J Exp Biol 121: 301–314

Molinas M Huguet G (1993) Ultrastructure and cytochemisrty of secretory cells in the sin of the leech, *Dina linesta*. J Morphol 216: 295–304

Ryder TA, Brown ID (1977) Endocytosis and aspects of autophagy in the foot epithelium of the slug *Agriolimax reticulatus*. Cell Tissue Res 181: 129–142

Simkiss K, Wilbur KM (1977) The molluscan epidermis and its serections. Symp Zool Soc London 39: 35–76

Skidmore R, Rivera ER (1982) Cytochemistry of the long-necked cells in the foot of *Onchidorus muricata* (Nudibranchia). Biol Bull (Woods Hole Mass) 162: 113–123

Tripp MR (1992) Agglutinins in the hemolymph of the hard clam, *Mercenaria mercenaria*. J Invertebr Pathol 59: 228–234

Yamaguchi K, Furuta E, Nakamura H (1999) Chronic skin allograft rejection in terrestrial slugs. Zool Sci 16: 485–495

Yuasa HJ, Furuta E, Nakamura A, Takagi T (1998) Cloning and sequencing of three C-type lectins from body surface mucus of the land slug, *Incilaria fruhstorferi*. Comp Biochem Physiol B119: 479–484

Zylstra U (1972a) Histochemistry and ultrastructure of the epidermis and the subepidermal gland cells of the freshwater snails *Lymnaea stagnalis* and *Biomphalaria pferifferi*. Z Zellforsch 130: 93–134

Zylstra U (1972b) Distribution and ultrastructure of epidermal and sensory cells in the freshwater snails *Lymnaea stagnalis* and *Biomphalaria pferifferi*. Neth J Zool 22: 283–298

(Received April 11, 2000 / Accepted June 15, 2000)