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Paragonimus ohirai Juveniles: Ultrastructural, Cytochemical and Autoradiographic Studies on the Development of the Gastrodermal Epithelium

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Abstract

Ultrastructural, cytochemical and autoradiographic studies were carried out on developing gastrodermis in *Paragonimus ohirai* juveniles. For comparative ultrastructural studies of the gastrodermis, some developmental stages of *P. westermani* were examined. The gut in newly excysted metacercaria was a tubular structure made of flattened epithelial cells with short simple cytoplasmic projections. During the course of development epithelial cells increased in volume and cytoplasmic projections extended to form lamellae which were occasionally bifurcated. The cytoplasm of the cells 5–15 days postinfection (p.i.) was marked by well-developed granular endoplasmic reticulum with dilated cisternae. Acid phosphatase (AcPase) could be differentiated in the cells 5 days p.i. by cytochemical tests, and secretory granules appeared at this stage. Autoradiography using ^{3}H-leucine showed that protein synthesis in the cells was intense 5–15 days p.i. These facts suggest that active metabolism for secretion and absorption occurred in the gastrodermal cells during juvenile growth. Ultrastructural and cytochemical observations showed that the gastrodermis of the *Paragonimus* species was almost adult-like by 15 days p.i.

Key words: Ultrastructure, cytochemistry, autoradiography, *Paragonimus ohirai* juvenile, *P. westermani* juvenile, gastrodermis

Introduction

Robinson and Threadgold (1975) stated that the gastrodermal cytoplasm of *Fasciola hepatica* adults has an ultrastructure which reflects its metabolic activity, secretion and absorption. Similar ultrastructural differences were also observed in gastrodermal cells of *Paragonimus westermani* and *P. ohirai* adults (Fujino and Ishii, 1988). Cytochemical observations demonstrated variations in the intensity of AcPase and Mg-ATPase activity in each cell. Cells in the secretory phase had denser reaction products than those in the absorptive phase (Fujino *et al.*, 1983; Fujino and Ishii, 1988).

Several ultrastructural studies of gastrodermal cells in juvenile stages of *F. hepatica* (Bennett and Threadgold, 1973; Bennett, 1975) and *Schistosoma mansoni* (Bogitsh and Carter, 1977) have been conducted. Bennett (1975) investigated development of the gastrodermal epithelium during migration in the host and described ultrastructural changes that occurred in epithelial cells during development. Bogitsh and Carter (1977) studied the development of the digestive tract of schistosomules grown *in vitro*. Fujino *et al.* (1983) compared cytochemical reactions for some phosphatases between juvenile and adult *F. hepatica*. They found weaker phosphatase reactions in juveniles, indicating that metabolic activity is less than in adults.

The present paper describes ultrastructural, cytochemical and autoradiographic studies of physiological and metabolic activities in gastrodermal cells during the development of *P. ohirai* juveniles. For comparative ultrastructural studies of the gastrodermis, some developmental stages of *P. westermani* were examined.

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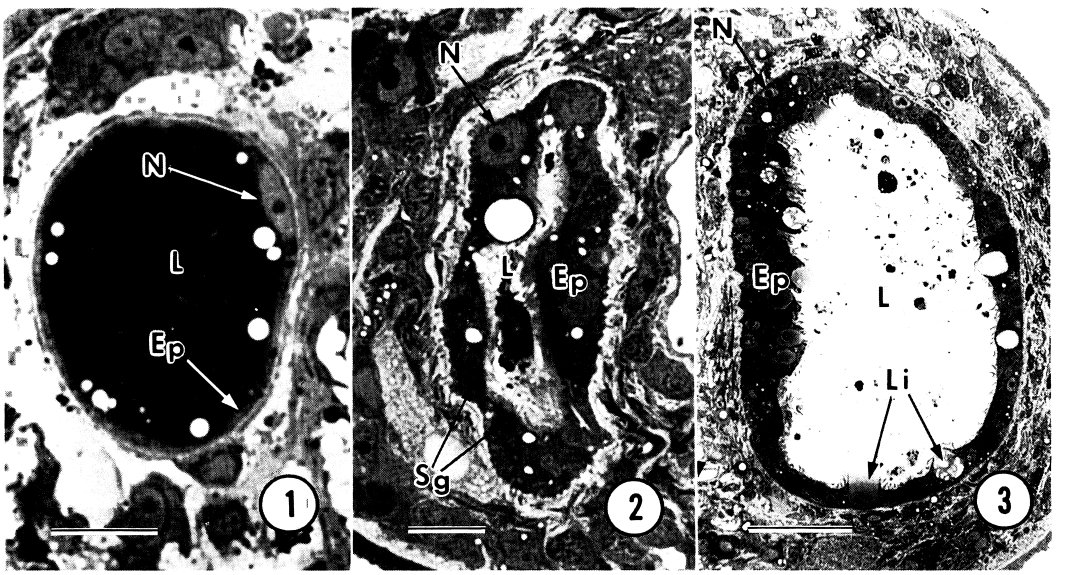
Materials and Methods

Metacercariae of *Paragonimus ohirai* were

removed from crabs, *Sesarma* (*Halometopus*) *dehaani*, taken in the Maruyama River, Hyogo Prefecture, central Japan. Albino rats (Sprague-Dawley) were infected orally, each with 50 metacercariae. Two rats were sacrificed to recover juveniles at 12 hr and on days, 1, 5, 10 and 15 after infection. Excystation of metacercariae was done at 39°C in 1% pancreatin plus 0.1% $NaHCO_3$. Metacercariae of *P. westermani* were obtained from crabs, *Eriocheir japonicus*, collected in the Tsushima Islands, western Japan. Dogs were orally infected with 50 metacercariae and then sacrificed on days 5 and 15 after infection. Juveniles were recovered from the abdominal cavity and liver of the infected dogs, respectively. Excystation of metacercariae was carried out by treatment with 0.07% HCl, followed by incubation in 0.1% $NaHCO_3$ in Ringer's saline at 39°C.

For transmission electron microscopy (TEM), worms were rinsed in Ringer's saline and fixed for 3 hr in 3% glutaraldehyde in 0.1 M phosphate buffer (pH 7.3) or in Karnovsky's (1965) fixative, and postfixed for 3 hr in phosphate buffered 1% osmium tetroxide (pH 7.3). After dehydration through an ethanol series, the material was embedded in Epon 812 or Quetol 812 (Nisshin EM, Tokyo). Thick sections were cut and stained with Toluidine Blue 0 and examined under the light microscope (LM). Thin sections were double stained with uranyl acetate and lead acetate and viewed in a Hitachi HS-9 electron microscope at 75 kV.

For AcPase cytochemistry, newly excysted metacercariae and juveniles of *P. ohirai* were removed on days 5 and 15 p.i. from rats, washed in Ringer's saline, and then fixed for 1 hr at 4°C in 2% glutaraldehyde plus 3% sucrose buffered with 0.1 M sodium cacodylate to pH 7.4. The tissues were sectioned with a McIlwain Tissue Chopper or an AO 975-Histostat set at 25–33 μm. The sections were collected in the buffer and then placed in the incubation medium for 1 hr at 37°C. Reaction for AcPase was examined by the modified lead nitrate method of Gomori (1952). Control incubations were performed with

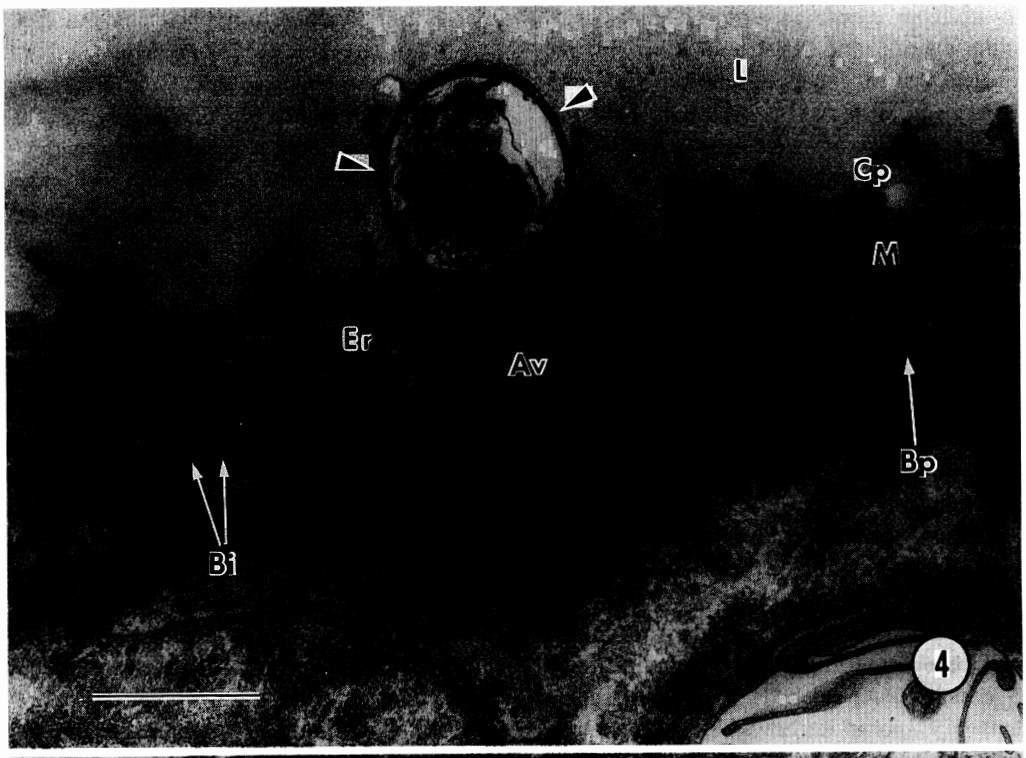


Figs. 1–3 *Paragonimus ohirai*. LM. Cross section of the gut. Stained with Toluidine Blue 0.

Fig. 1 Newly excysted metacercaria. The gut is round and lined with thin epithelial cells containing large flattened nuclei. The luminal content is dense. Bar = 10 μm.

Fig. 2 5 days p.i. The gut is elongated and the epithelium is thicker and well defined. Some dense secretory granules appear in the cells. Bar = 10 μm.

Fig. 3 15 days p.i. The epithelium is well developed and adult-like. Bar = 50 μm.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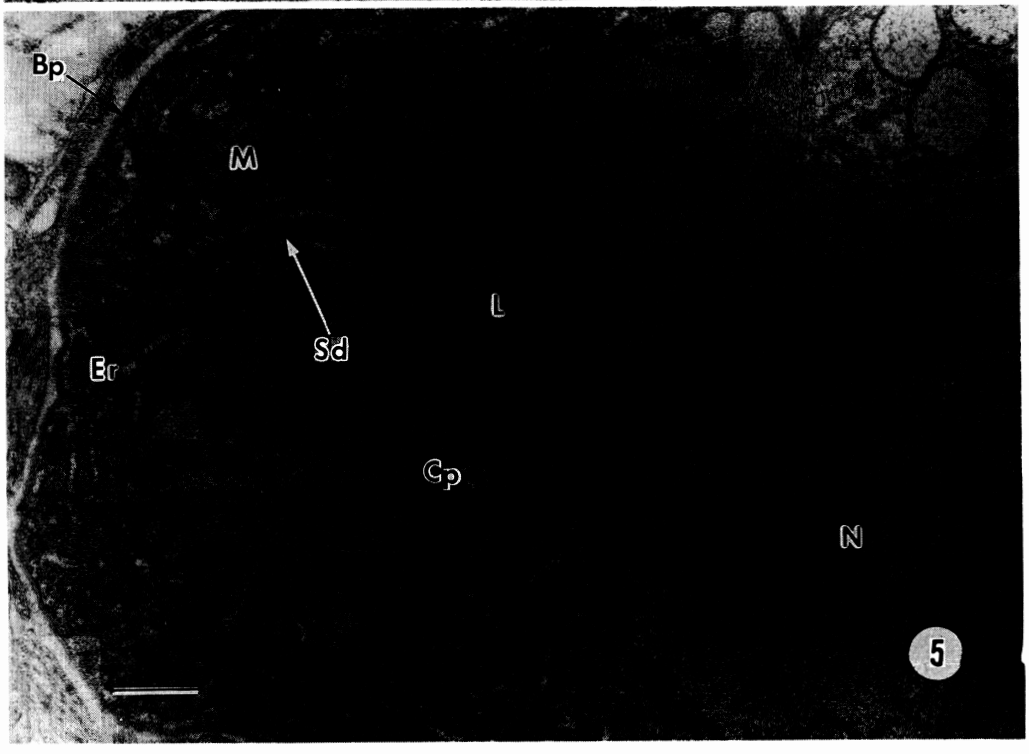
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the inhibitor, 10mM sodium fluoride. After a brief rinse in buffer, the tissues were postfixed, dehydrated and embedded as mentioned above. Sections were examined unstained or after brief staining with uranyl acetate.

For autoradiography, tritiated leucine was used as a marker for protein synthesis. L-[3,4,5-^{3}H]- leucine with a specific activity of 2.22 TBq/mmol, was obtained from CEA, France. Newly excysted metacercaria and 5 and 15-day-old juveniles of *P. ohirai* were examined. Adult worms (60-day-old) were also tested for comparison. The worms were pulse labeled for 1 hr at 37°C in NCTC 135 medium including 3.7×10^6 Bq/ml of tritiated leucine and 10% rat (SD) serum plus 100 iu/ml streptomycin and 100 ug/ml penicilin. After pulse incubation the worms were washed in several changes of NCTC 135 medium, and then separated into three groups for chase incubation at 0, 5 and 24 hr. The chase incubation in NCTC 135 medium without labeled substrate was carried out at 37°C in a CO_2 incubator (95% air, 5% CO_2). At the end of each chase incubation, worms were washed in 0.1 M phosphate buffer, fixed for 2 hr in 6% phosphate-buffered glutaraldehyde, and then embedded for TEM as described earlier. Thick sections (1 μm) were cut and transferred to glass slides. They were dipped into a solution of Konica NR-M2 emulsion that was mixed 1:1 (w/v) with water. Slides were dried, and stored at 4°C in a light-tight container. After 3 weeks, the slides were developed for 5 min at 20°C, washed in cold water, fixed in a solution of Konicafix for 15 min, stained with Toluidine Blue 0, and examined.

Results

LM and TEM observations in *P. ohirai*:

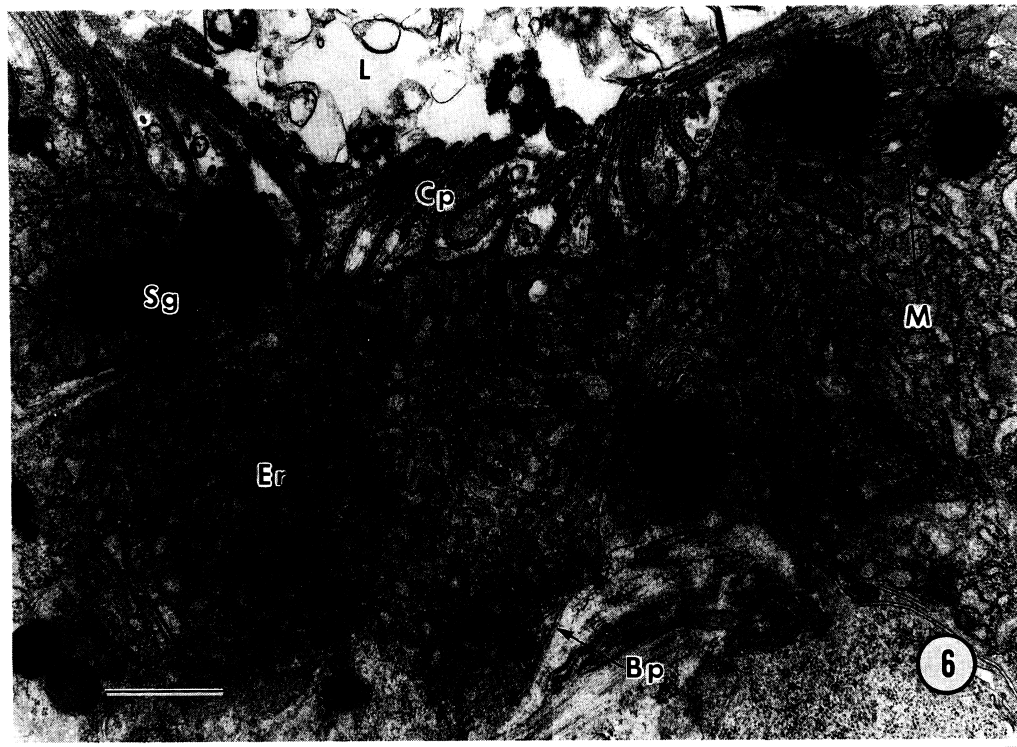
Metacercaria — In cross section, the gut was round and lined by a thin epithelium which was thicker in areas where large nuclei protruded into the lumen (Fig. 1). The lumen was filled with dense uniform material. Junctions between cells were marked by a prominent septate desmosome. At the border between cells lateral plasma membranes were strongly curved or meandered. The cytoplasm was filled with ribosomes and contained some granular endoplasmic reticulum. Mostly round or oval mitochondria with a few short cristae were distributed throughout the cytoplasm. Nuclei were flattened and occupied a large area of the cells. Short simple cytoplasmic projections were sparsely located on the surface of the epithelium. The basal plasma membrane, which was straight or gently curved, formed occasional basal invaginations.

12 hr p.i. — In cross section, the gut was elongate with a broadened or partly narrowed lumen. Granular endoplasmic reticulum was more abundant, and many ribosomes formed polysomes. Mitochondria were occasionally elongate. Nuclei were flattened or oval. Cytoplasmic projections were short, mostly simple and rarely bifurcated.

1 day p.i. — The epithelium was thicker with an undulating basal plasma membrane. The cytoplasm was marked by the development of granular endoplasmic reticulum with moderately dilated cisternae (Fig. 5). Oval nuclei, which occupied a large area of the cytoplasm, projected into the lumen. Small round or oval mitochondria with short cristae were dispersed in the cells. Cytoplasmic projections were longer, mostly simple and rarely bifurcated. Surface linear structures had not yet developed. Ingested blood cells and food debris were seen in the lumen. Basal invaginations were developed partly in the cells.

Fig. 4 *P. westermani*, newly excysted metacercaria. Thin epithelial cells have cytoplasm which contains round or oval mitochondria with short cristae and partly developed granular endoplasmic reticulum. Cytoplasmic projections are short, simple and irregularly placed. The basal plasma membrane forms occasional basal invaginations. Arrowheads indicate a superficial vacuole which is rarely formed by the cytoplasmic projections on the apical plasma membrane. Bar = 1 μm.

Fig. 5 P. *ohirai*, 1 day p.i. The epithelium is thicker than in metacercaria and has an undulating basal plasma membrane. Granular endoplasmic reticulum has dilated cisternae. A large nucleus is seen. Lamellar cytoplasmic projections are longer and more numerous. Bar = 1 μm.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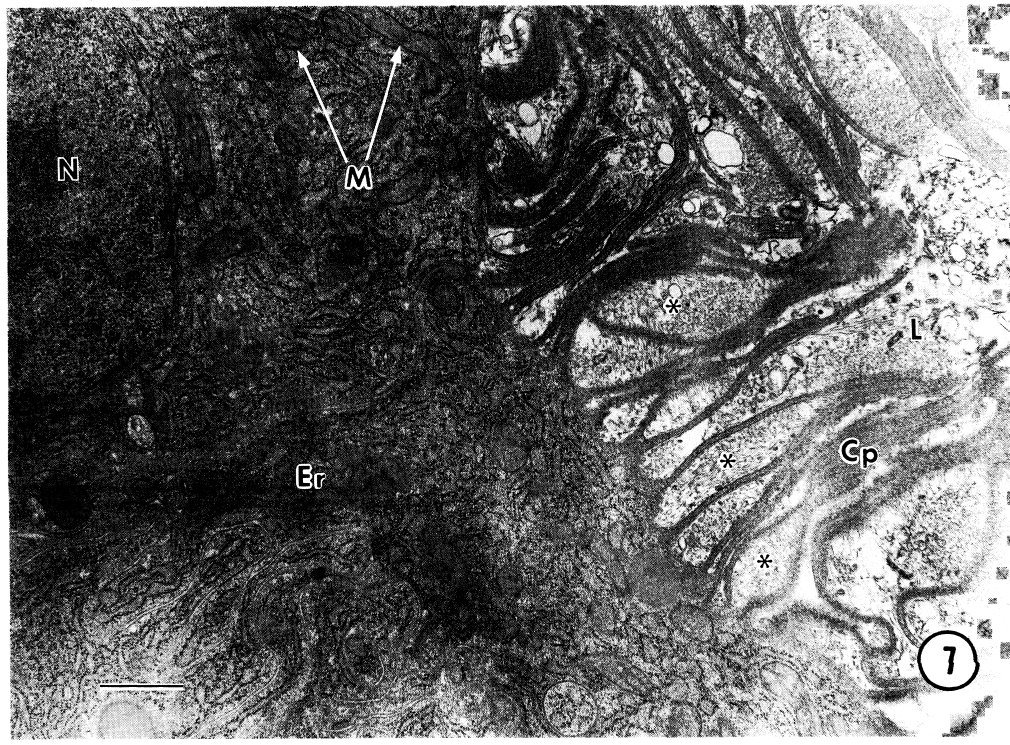
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5 days p.i. — Epithelial cells were well defined (Figs. 2, 6). The cytoplasm was filled with well-developed granular endoplasmic reticulum with dilated cisternae (Fig. 6). Dense secretory granules and lipid droplets were observed in some cells. Cytoplasmic projections increased in number and had lengthened.

10 days p.i. — Epithelial cells were thicker than at 5 days p.i. and the cytoplasm contained abundant granular endoplasmic reticulum with extensive cisternae (Fig. 7). Some cells had secretory granules, while others occasionally contained large cytoplasmic bodies. Cytoplasmic projections had undergone further extensions and basal invaginations were present. Linear tubular structures had developed on the epithelial surface or between lamellar cytoplasmic projections. Large quantities of food particles were entrapped between the projections.

15 days p.i. — The gut epithelium at this stage was similar to those of adults (Fig. 3) and cells could be classified as either absorptive or secretory. Epithelial cells in the secretory phase contained a number of secretory granules, while the cells in the absorptive or transitional phase were devoid of these inclusions (Fig. 8). Small oval mitochondria were dispersed throughout the cytoplasm. Phagosomes containing luminal food particles occurred near the apical plasma membrane. Cytoplasmic projections were long and had occasional bifurcations.

TEM observations in *P. westermani*:

The gut epithelium of metacercaria was thin with a smooth basal plasma membrane. There were elongate granular endoplasmic reticulum and round mitochondria in the cytoplasm. Autophagic vacuoles and groups of glycogen particles were seen occasionally. Superficial digestive vacuoles were rarely found on the epithelial surfaces of the cells (Fig. 4).

The epithelium at 5 days p.i. was full of granular endoplasmic reticulum with a smooth basal plasma membrane. A large number of small non-electron dense secretory granules were found in the cytoplasm. The lumen was filled with dense material. Nuclei were flattened and oval, being situated close to the bases of the cells.

In the epithelium at 15 days p.i., autophagic vacuoles were observed in some cells (Fig. 9). Small round or elongate mitochondria with short and sparse cristae were dispersed throughout the cytoplasm.

Cytochemical observations (AcPase):

Metacercaria — No marked reaction products were present in epithelial cells except small occasional deposits.

5 days p.i. — Some differences in enzyme reaction were evident in gastrodermal cells (Fig. 10). In some cells, granular material as well as larger round reaction deposits were dispersed throughout the cytoplasm. Some deposits were associated with lysosomal bodies. Reaction deposits lay irregularly on the surface of the lamellar cytoplasmic projections. In the other cells, no or few reaction deposits occurred in the cytoplasm.

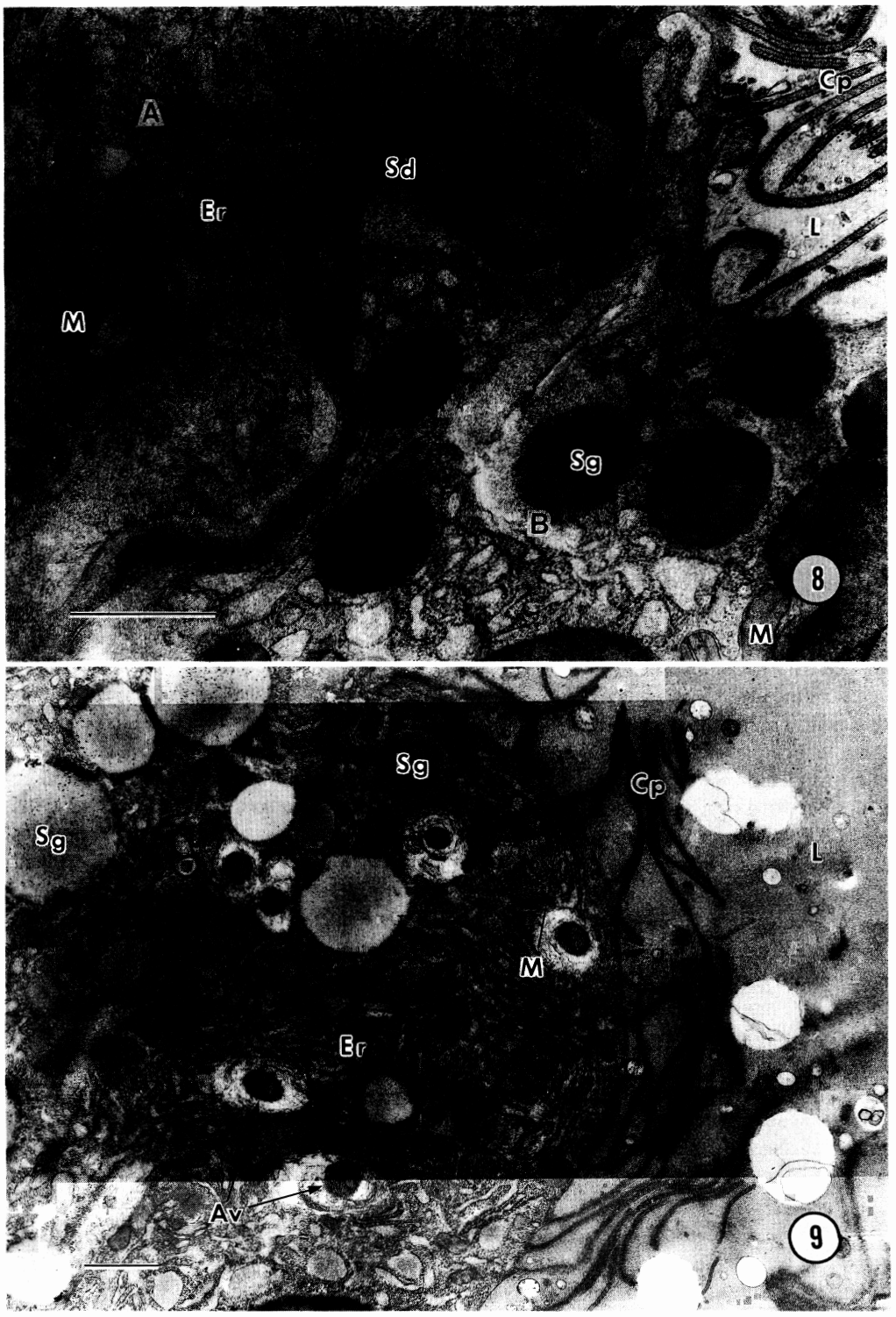
15 days p.i. — Clear differences in enzyme reaction were seen in cells (Fig. 11). Cells with many secretory granules had intense reaction deposits in the cytoplasm. Within individual cells, basal regions with thicker secretory granules had more intense reactions than apical regions with fewer secretory granules. Cells that possessed cytoplasmic bodies and few secretory granules had weak reactions. Some reaction deposits occurred around or in lysosomal bodies.

Autoradiography (^{3}H-leucine):

Metacercaria — Some silver grains were observed on the gut epithelium after 1 hr of pulse

Fig. 6 *P. ohirai*, 5 days p.i. The epithelium is much thicker than that at 1 day p.i. The cytoplasm is filled with granular endoplasmic reticulum with dilated cisternae. Small round mitochondria are dispersed throughout the cytoplasm. Some secretory granules are present. Cytoplasmic projections extend and bear linear tubular structures on their surfaces. Bar = 1 μm.

Fig. 7 *P. ohirai*, 10 days p.i. The epithelium is characterized by well-developed granular endoplasmic reticulum. The cytoplasmic projections are longer and form superficial enclosed spaces (*), in which food is held or entrapped. Bar = 1 μm.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label (Fig. 12). Sections after 5 and 24 hr of chase incubation showed decreased radioactivity.

5 days p.i. — Single silver grains were thickly dispersed over the cells after 1 hr of pulse label. Sections processed after 5 hr of chase showed increased concentrations of radioactivity (Fig. 13). Radioactivity in the epithelium decreased after the 24 hr chase.

15 days p.i. — Incorporation of leucine into the epithelium was most active around 5 hr chase. Small clusters of silver grains as well as single grains were distributed throughout the epithelium (Fig. 14). Silver grains were few in the lumen. Radioactivity in the cells was comparatively weak after 24 hr of chase.

Adult (60 days p.i.) — Numbers of silver grains were fewer than at 5–15 days p.i. after either 0, 5 or 24 hr of chase (Fig. 15). Label appeared in association with secretory granules. Only a few numbers of silver grains were seen in the cells after 24 hr of chase.

Discussion

There appeared some ultrastructural differences in the gastrodermal epithelium during the development between *Paragonimus ohirai* and *P. westermani* juveniles. The epithelium of *P. westermani* is thicker than that of *P. ohirai.* In the metacercaria, autophagic vacuoles, glycogen particles and superficial vacuoles are occasionally found in *P. westermani.* These structures are rare in *P. ohirai* and the cytoplasm is characterized by lots of ribosomes. In 5 days p.i., a large number of non-electron dense secretory granules are present in *P. westermani,* whereas granules are fewer and dense in *P. ohirai.*

Gut epithelial cells in *P. ohirai* increased in thickness as worms developed in the definitive host. The cytoplasm of cells at 5–15 days p.i. contained well-developed granular endoplasmic reticulum with dilated cisternae and secretory granules. This indicates that protein synthesis became active during early stages of juvenile development. AcPase activity was first detected at 5 days p.i. and exhibited regional differences in gastrodermal cells. AcPase was reported to be involved in the secretion and/or digestion of foodstuffs (Threadgold, 1968; Lumsden, 1975). This suggests that differences in metabolic activity in the gastrodermal cells of *Paragonimus ohirai* occurred for the first time at this stage of development.

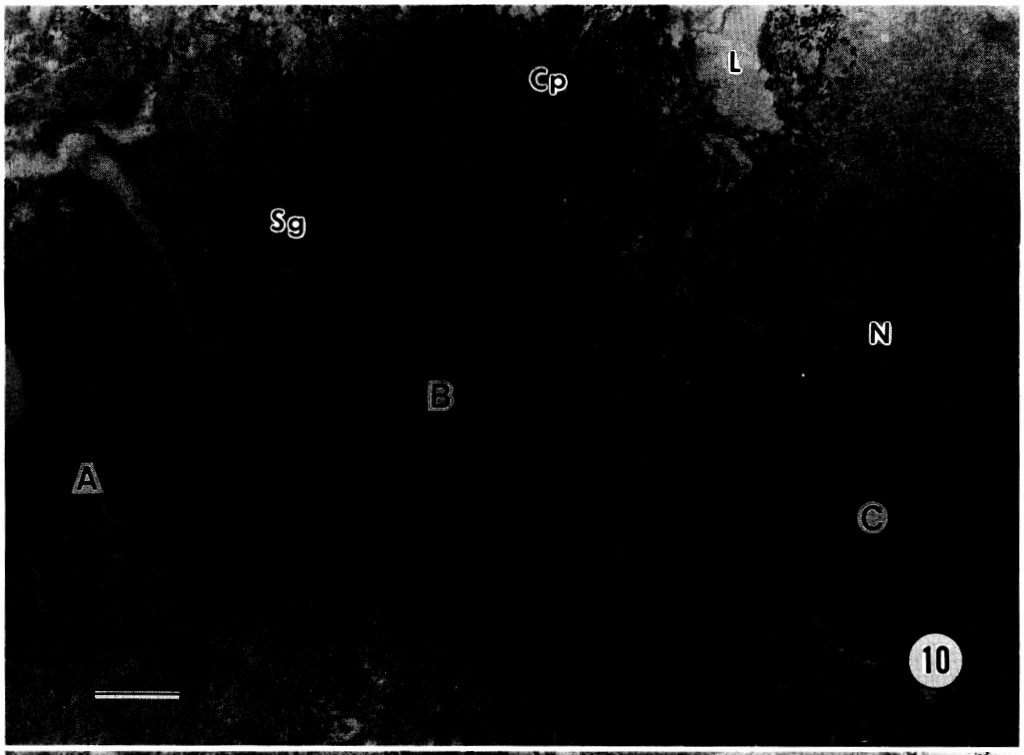
The epithelial structure of worm at 15 days p.i. was similar to that of adults. Many cells contained abundant secretory granules and had intense AcPase activity, while the other cells with lysosome-like cytoplasmic bodies and few or no secretory granules had weak AcPase activity. The former cells were probably in the secretory phase and the latter in the absorptive or the transitional phase between the secretory and absorptive phases. Variations in ultrastructure and cytochemistry may reflect different physiological conditions in the cells. A secretory-absorptive cycle has been described in the gastrodermal cells of *Paragonimus* adults (Fujino and Ishii, 1988), *Fasciola hepatica* adults (Threadgold, 1968) and young stages of *F. hepatica* (Fujino *et al.*, 1983).

It is possible that the beginning of active secretion from epithelial cells at 5–15 days p.i. is associated with the development of host immune responses. It has been pointed out that granules secreted by gastrodermal cells are involved in intraluminal digestion (Thorsell and Björkman, 1965; Halton, 1967) and are probably antigenic. Ohara *et al.* (1985) reported that levels of serum IgG antibody against the gut as well as the tegument from rats infected with *P. ohirai* increased gradually as juvenile worms developed and reached a peak at 8 weeks p.i.

In the newly excysted metacercaria and very young stages of *F. hepatica,* the cytoplasm of

Fig. 8 *P. ohirai,* 15 days p.i. Secretory-absorptive phases are clearly defined by the presence of secretory granules in cells A and B, which are joined by an apically located septate desmosome. Cell B contains many secretory granules, while A has few. Bar = 1 μm.

Fig. 9 *P. westermani,* 15 days p.i. The cell contains many autophagic vacuoles as well as secretory granules. Bar = 1 μm.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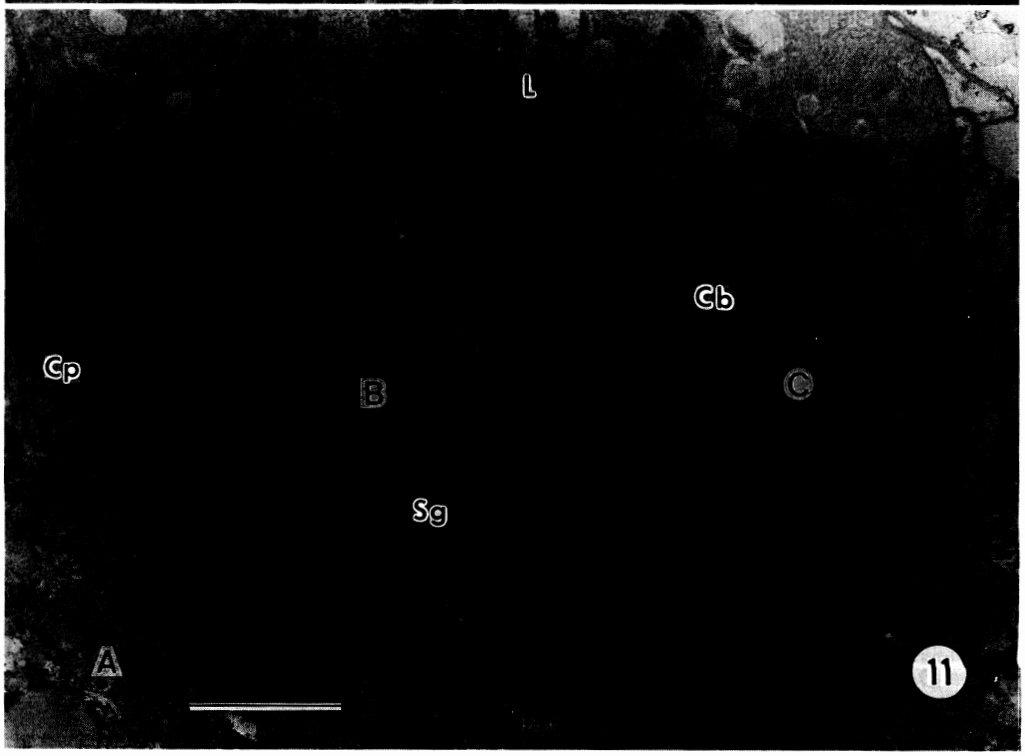
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gastrodermal cells is dominated by large ovoid and very dense secretory granules, and these granules are released from the gastrodermis as the worms migrate across the host intestinal wall (Bennett and Threadgold, 1973; Bennett, 1975). Bennett and Threadgold (1973) and Bennett (1975) speculated that the secretory granules disrupt host cells and facilitate penetration in the host. *Paragonimus* species, in contrast, have no secretory granules in such young stages and secretory granules are produced around 5 days p.i. It therefore is possible that invasive way to the hosts is different between these species.

Autoradiographs demonstrated that gastrodermal cells are major sites for radioleucine incorporation and protein synthesis. Even cells of newly excysted metacercariae showed some labeling. Incorporation of leucine and concurrent protein synthesis appeared very active 5–15 days p.i., because of large numbers of silver grains that formed clusters over the cells. Incorporation of leucine in adults was less than that of juveniles, that were examined at corresponding chase incubation periods. These facts may correspond to cytochemical observations indicating that enzyme activities in the gastrodermal cells were most active in the juvenile stages, especially 5–15 days p.i. Wilson and Barnes (1979) also used tritiated leucine as a marker for protein synthesis for the study on the synthetic and secretory capacity of the tegument, gut and nephridial epithelia of *Schistosoma mansoni*. They noted that labeled protein disappears from the gut lumen relatively rapidly and that the half-life of secretory protein in the gut cells is around 2 hr. In the present study, the labeled protein appeared after 1 hr of pulse label. The specific activity reached a peak around 5 hr of chase incubation and then decreased by 24 hr incubation.

Fujino *et al.* (1987) demonstrated numerous linear tubular structures on the surfaces of the lamellar cytoplasmic projections in *Paragonimus* species. They noted that these structures are probably made of glycosaminoglycans and may adsorb food particles to help in their digestion and absorption. These structures were thought to originate from the gastrodermal cells (Fujino *et al.*, 1987). The present study failed to detect these structures on short simple cytoplasmic projections in the newly excysted metacercariae. Traces of these structures were observed by 1 day p.i., and they became well developed by 5 days p.i., and more elaborate as worms matured. The above observations suggest that the tubular structures originate from gastrodermal secretions, although the mechanism of their formation is not clear.

Acknowledgements

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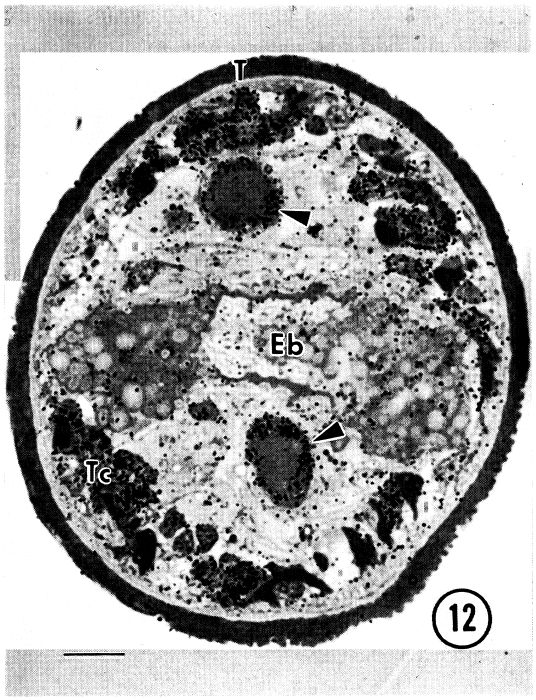
Figs. 10 and 11 *P. ohirai*. Cytochemical reaction for AcPase.
Fig. 10 5 days p.i. Reaction deposits are marked in cell B. Some deposits are associated with lysosomal bodies. The cytoplasmic projections have granular deposits on their surfaces. No deposits are seen in cells A and C. Bar = 1 μm.
Fig. 11 15 days p.i. An intense enzyme reaction is in cell B which contains numerous secretory granules. Cell C has few secretory granules and many cytoplasmic bodies and shows a weak enzyme reaction. Bar = 3 μm.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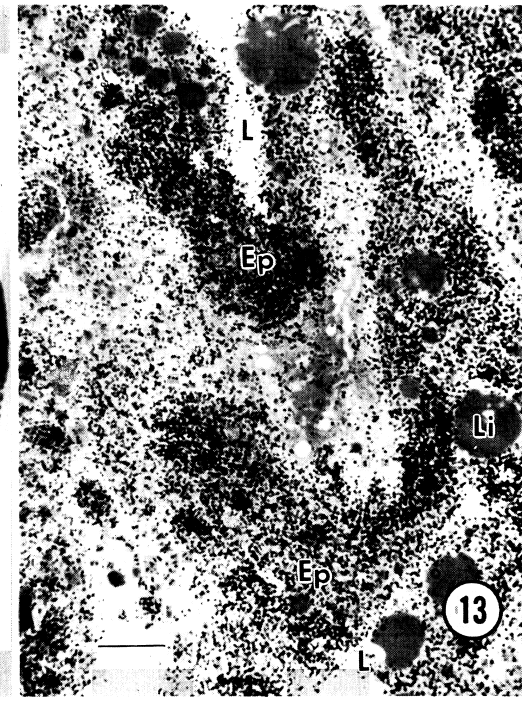
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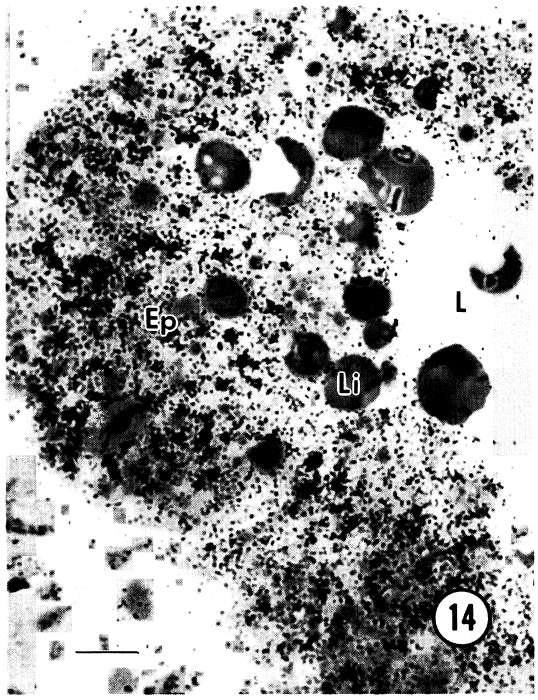
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Figs. 12–15 *P. ohirai* Autoradiographs of ^{3}H-leucine incorporation in the gut epithelium. Bar = 10 μm.

Fig. 12 Metacercaria, 1 hr of pulse label. Cross section of body. Some silver grains are labeled over the gut epithelium (arrowheads).

Fig. 13 5 days p.i. 1 hr of pulse label followed with 5 hr of chase in nonradioactive leucine. Radioactivity is concentrated in the cells.

Fig. 14 15 days p.i. 1 hr of pulse followed with 5 hr of chase in nonradioactive leucine. A number of small clusters of silver grains as well as single grains are dispersed over the cells.

Fig. 15 Adult (60 days p.i). 1 hr of pulse followed with 5 hr of chase in nonradioactive leucine. The density of silver grains over the cells is less than that of juveniles.

Key to lettering of figures

Av : Autophagic vacuole
Bi : Basal invagination
Bp : Basal plasma membrane
Cb : Cytoplasmic body
Cp : Cytoplasmic projection
Eb : Excretory bladder
Ep : Gut epithelium
Er : Granular endoplasmic reticulum
L : Lumen
Li : Lipid droplet
M : Mitochondrion
N : Nucleus
Sd : Septate desmosome
Sg : Secretory granule
T : Tegument
Tc : Tegumental cell