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Expression of a Tegumental Antigen during the Development of *Paragonimus ohirai*

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Abstract

Monoclonal antibodies were developed against tegumental glycoproteins from metacercariae of *Paragonimus ohirai*. We used one of these antibodies and postembedding immunoelectron microscopy to localize antigenic sites that were associated with the tegument of metacercariae, 5-, 15-, 25-, and 50-day-old worms. Four types of tegumental granules were identified. These included moderately electron-dense G0 granules which were only observed in metacercariae, round, electron-dense G1 granules which occurred in all stages of development, and two types of discoidal or oval-shaped G2 granules (G2L and G2D) which appeared in 25-day-old worms and persisted as the worms matured. G2L and G2D granules were morphologically similar, but differed in electron density and antigenicity. We found extensive immunolabeling in association with G0, G1, and G2L granules and the tegumental plasma membrane of metacercariae, 5-, 15-, and 25-day-old worms. Label was not detected in G2D granules or the tegumental plasma membrane of mature 50-day-old worms. Our findings suggest that antigens associated with the glycocalyx are packaged in various tegumental granules and expressed continuously on the surface of migrating juvenile worms as a means of avoiding immunological attack by the host.

Key words: *Paragonimus ohirai*, tegumental antigens, monoclonal antibodies, immunoelectron microscopy

Introduction

In *Fasciola hepatica*, there are two distinct types of tegumental cells from which different types of granules are produced (Bennett and Threadgold, 1975; Hanna, 1980a; Hanna and Trudgett, 1983). Both of the granules are probably responsible for the formation of surface antigens, and glycocalyx turnover may help protect migrating juvenile worms against immunological attack.

Several mechanisms may operate to protect adult schistosomes. Absorption of host antigens may mask worms from immunological recognition (Smithers *et al.*, 1969). It is also known that the heptalaminate tegumental surface may be capable of rapid renewal (Hockley and McLaren, 1973; Perez and Terry, 1973; Kusel *et al.*, 1975; Wilson and Barnes, 1977; Aronstein *et al.*, 1983).

In the present investigation, monoclonal antibodies were developed against tegumental glycoproteins from newly excysted metacercariae of *Paragonimus ohirai*. We have used an immunocolloidal gold method to trace changes in the distribution of these immunogenic glycoproteins during the development of metacercariae to adults.

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Materials and Methods

Preparation of specific antigen

Crabs, *Sesarma* (*Halometopus*) *dehaani*, that were infected with metacercariae of *Paragonimus ohirai* were collected from the Maruyama River,

Hyogo Prefecture, central Japan. Metacercariae were allowed to excyst during an overnight incubation in Tyrode's solution, pH 8.0 at 37°C. Newly excysted metacercariae were sonicated in phosphate buffer (pH 7.2). After centrifugation at 20,000 g for 30 min, the supernatant was freeze-dried, and used as the crude antigen.

Development of monoclonal antibodies

BALB/c mice were immunized by the subcutaneous injection of 1.0 mg crude antigen which had been emulsified in Freund's Complete Adjuvant. Booster immunizations were carried out by intraperitoneal injections of 0.5 mg of crude antigen. Spleens from immunized mice were removed 4 days after the last booster immunization. Splenic lymphocytes were fused with cultured P3UI myeloma cells with polyethylene glycol. Spleen x myeloma hybrids were selected with hypoxanthin/aminopterin/thymidine (HAT) medium in microtitre trays. After 2 weeks of culture in RPMI 1640 medium plus 10% FBS and 25 mM HEPES buffer, the medium from each well was screened by enzyme-linked immunosorbent assay (ELISA) using peroxidase conjugated anti-mouse IgG for the presence of IgG antibody against worm tissues. Hybridoma cell lines producing a specific antibody (IgG2b) were cloned by limiting dilution, and the antibody was subsequently purified from ascitic fluid.

Characterization of antigen

The antigen resisted heat (100°C, 10 min), but was inactivated by the treatment of 0.05 M periodic acid (4°C, 1 hr), 0.5% Pronase or 0.25% trypsin (37°C, 10 hr). The antigen showed a strong affinity with peanut lectin (PNA) and a weak affinity with *Ricinus communis* (RCA) lectin. The presence of tegumental antigen was confirmed by immuno-diffusion against anti-tegumental glycoprotein monoclonal antiserum. The monoclonal antibody was examined to recognize the antigen of molecular weight over 669 K dalton by gel filtration using a high pressure liquid chromatography.

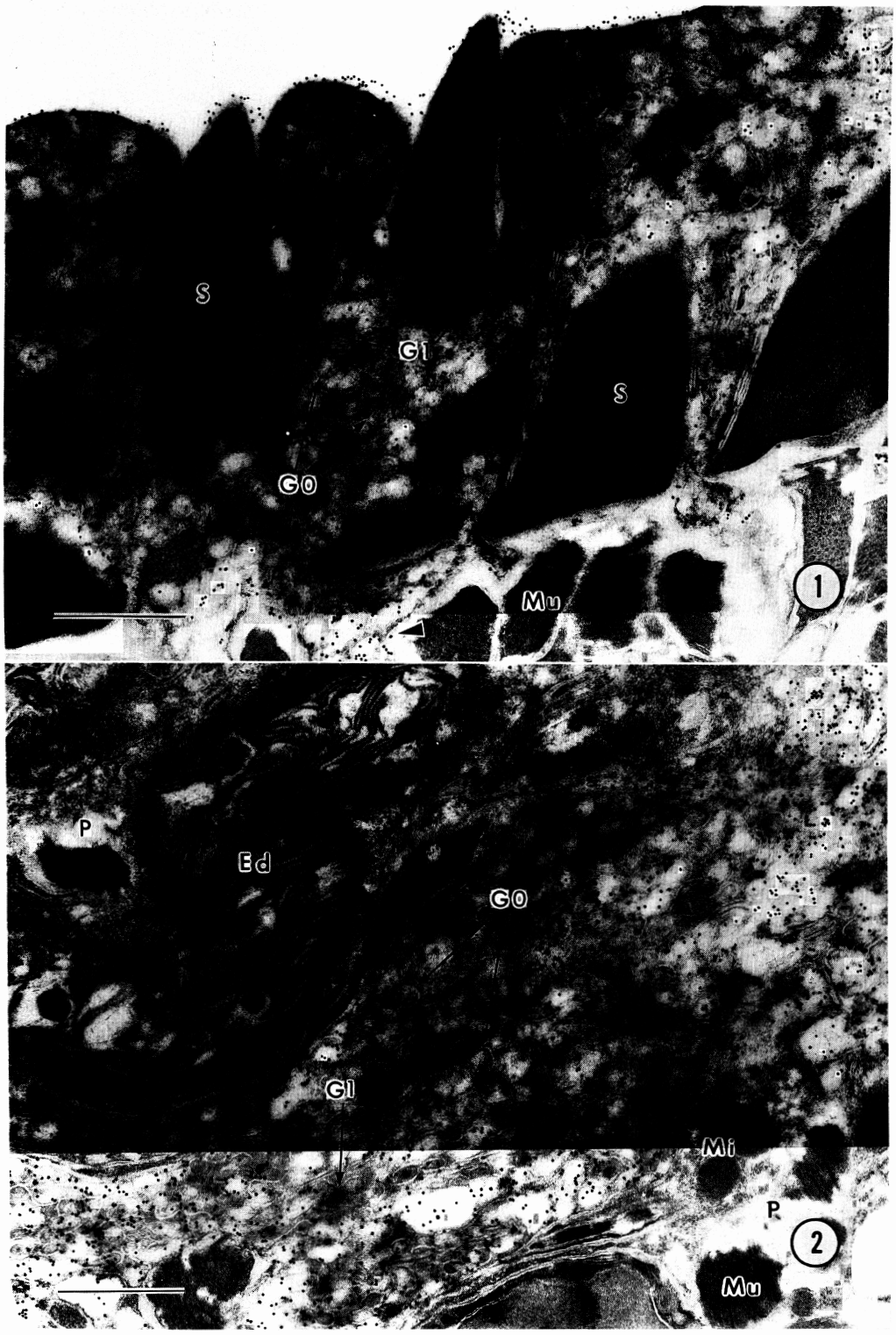
Ultrastructural localization of antigen

Newly excysted metacercariae were prepared, and 5-, 15-, 25- and 50-day-old worms were removed from the abdominal cavity, liver, pleural cavity and lung of albino rats, respectively. These worms were cut into small pieces and fixed for 90 min at 4°C with 2% paraformaldehyde plus 0.3% glutaraldehyde in 0.1 M phosphate buffer (pH 7.3), containing 3% sucrose and 5×10^{-4} M $CaCl_2$. After several washes in phosphate buffer, the tissues were dehydrated in an ethanol series, infiltrated overnight with LR White resin (London Resin Co. Ltd., Basingstoke, England), and placed in gelatin capsules with fresh resin. The sealed capsules were polymerized at 55°C for 20 hr. Ultrathin sections were cut and picked up on unsupported 400 mesh nickel grids.

Prior to immunolabeling, the grids were floated on drops of 5% (w/v) nonfat dry milk in buffered saline to block nonspecific staining. After blocking the grids were immersed in antibody-containing ascites which had been diluted to 1/500 with 1% bovine serum albumin (BSA) in 0.1 M PBS with 0.001% tween-20 (PBS-BSA-tween). The grids were incubated for 2 hr at room temperature. Control sections were similarly incubated in non-immune mouse serum diluted to 1/500. After incubation, grids were rinsed with PBS-BSA-tween solution and incubated for 1 hr at room temperature in the second antibody (rabbit anti-mouse IgG) which had been diluted to 1/100 with PBS-BSA-tween. The grids were then washed with buffer and transferred to protein-A-gold (E-Y Laboratories Inc., San Mateo, Ca USA) diluted to 1/10 with PBS-BSA-tween. The grids were washed with dis-

Fig. 1. Tegument of a newly excysted metacercaria. G0 and G1 granules are labeled. Some gold particles are located over the tegumental plasma membrane. Labeling is absent on spines, mitochondria and the cytoplasm. Labeled G0 granules are also present in connecting tubules which link the surface syncytium to tegumental cells (arrowhead). Mu: Muscle; S: Spine. Bar = 1 μm.

Fig. 2. Tegumental cells of a newly excysted metacercaria. Labeling is concentrated on G0 granules. The excretory duct, muscles, and adjacent parenchymal cells are not labeled. Ed: Excretory duct; Mi: Mitochondrion; P: Parenchymal cell; Mu: Muscle. Bar = 1 μm.


S
Gl
S
GO
Mu
1
P
Ed
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Mi
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tilled water and the sections were stained for 10 min with 1% uranyl acetate and 5 min with lead acetate. The sections were examined at 75 kV with a Hitachi HS-9 electron microscope.

Electron microscopy (TEM)

Worms at each stage of development were removed from the host tissue, washed with 0.1 M phosphate buffer, dissected into small pieces and fixed in both 3% glutaraldehyde and 1% osmium tetroxide. After worms were embedded in Epon 812, sectioned, and stained with uranyl acetate and lead acetate, the grids were examined as described above.

Results

Newly excysted metacercaria

The tegument was filled with ovoid, moderately electron-dense granules (G0), and a small number of electron-dense, round granules (G1). Labeling occurred on almost all G0 and G1 granules (Figs. 1, and 8a). Gold particles were also localized on the apical plasma membrane of the tegument. No labeling occurred in the cytoplasm, spines or mitochondria. Granules in the cytoplasmic tubules leading from the tegumental cells to the tegument were also labeled. G0 granules located in the tegumental cells below the tegument were also labeled (Fig. 2). Excretory ducts, muscles, mitochondria and parenchymal cells were not labeled.

5 to 25-day-old worms

Discoidal or oval-shaped electron-dense granules (G2L) which contained a homogenous material were distributed densely throughout the tegument. These granules as well as G1 granules were labeled intensely (Figs. 3, 8b). No G0 granules were observed. As was observed in the metacercaria, labeling also occurred over the apical plasma membrane. Labeling was not seen on the tegument, mitochondria and spines. Less electron-dense granules (G2L) of the tegumental cells were labeled (Fig. 4). No labeling occurred on the nucleus, mitochondria or the cytoplasm of the tegumental cells.

50-day-old worms

Labeling was found on less electron-dense granules (G2L) in the tegument. Disk-like dense granules (G2D) were not labeled (Figs. 5 and 8c). Very little or no labeling occurred on the apical plasma membrane of the tegument. There appeared to be the tegumental cells that contained many randomly distributed labeled G2L granules and a few unlabeled G2D granules, while the others included numerous G2D granules with a few G2L granules (Fig. 6). Various types of the tegumental cells with different proportion of G2L and G2D granules were also evident by standard TEM (Fig. 7).

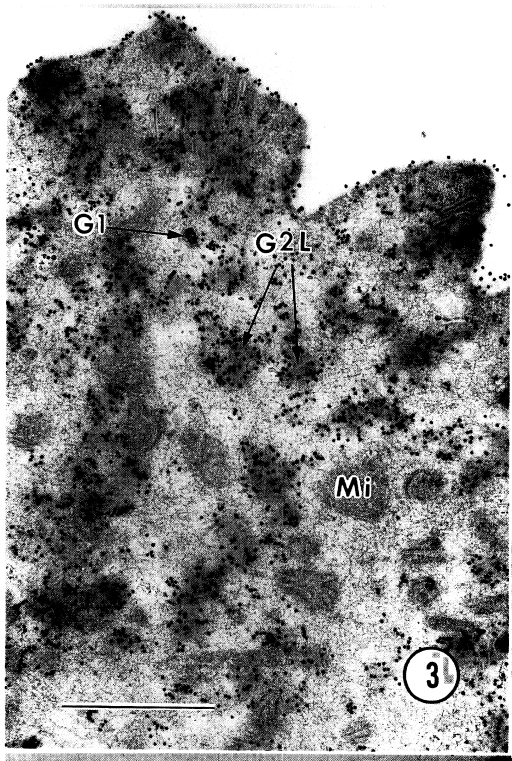
Discussion

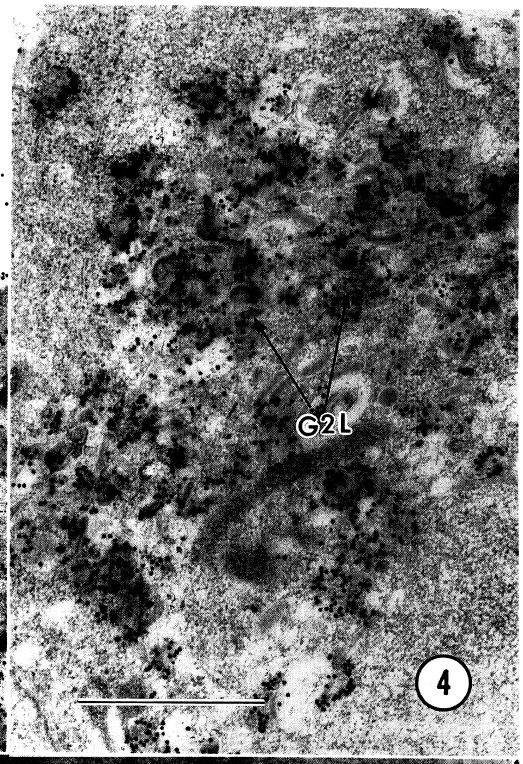
In the present study, we have used a monoclonal antibody that binds to tegumental granules of *P. ohirai* to trace the expression of these antigens during growth and development. Localization of antigen-antibody binding with an immuno-gold method confirmed that antigenic substances are located in G0, G1 and G2L granules in the tegument and tegumental cells. The G0 granules, specific to the metacercaria, change into G2L granules by 5 days postinfection. Although G0 and G2L granules are quite different morphologically from each other, they seem to possess antigenically similar glycoproteins. G1 granules are few in number and appear throughout development of metacercariae to adults. Hanna (1980a) also reported that morphologically different T0 and T1 granules from the tegument of juvenile *Fasciola hepatica* had a similar antigenic composition. In 5 to 50-day-

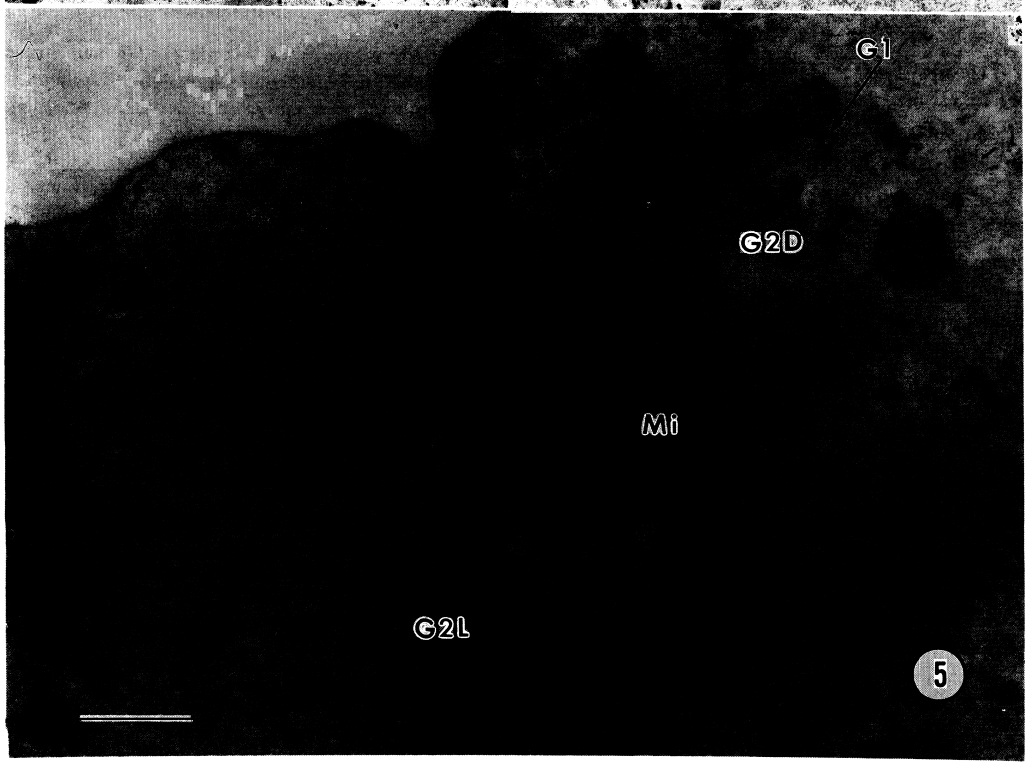
Fig. 3. Tegument of a 5-day-old worm. Specific label is present on granules. The cytoplasm is not labeled. Mi: Mitochondrion. Bar = 1 μm.

Fig. 4. Tegumental cell of a 15-day-old worm. G2L granules are labeled. Bar = 1 μm.

Fig. 5. Tegument of a 50-day-old worm. Less-dense G2L granules are labeled. The denser G2D granules are not labeled. Mi: Mitochondrion. Bar = 1 μm.


G1
G2L
Mi
3


G2L
4


G1
G2D
Mi
G2L
5

old worms both G2L and G2D granules can be found in the tegumental cells. These two types of granules are similar in shape although the G2L granules are less electron dense than G2D granules. G2D granules occasionally take the form of narrow discs. Since only G2L granules were labeled in our study, it seems likely that G2L and G2D granules differ in antigenicity. G2D granules first appear in the tegumental cells around 5 days postinfection and increase in number with the growth of the worms. According to Fukuda (1986), tegumental granules of adult *Paragonimus* species can be separated into four types. Of these G2-G4 granules show considerable overlap in morphology and should be classified as two types, G2L and G2D granules. In *F. hepatica,* T2 granules begin to accumulate in the tegument at 1 week of post-infection. These T2 granules that are originated from the different type of cells are antigenically different from T0 and T1 granules and appear to be produced continually throughout development (Bennett and Threadgold, 1975; Hanna, 1980a). In antigenic composition, T0 — T1 and T2 granules in *F. hepatica* appear to correspond to G0 — G2L and G2D in *P. ohirai,* respectively.

It is probable that G0 and G2L granules contribute to the formation of glycocalyx on the apical plasma membrane of the tegument. Labeling occurs over the plasma membrane of metacercariae and juveniles, but rarely or not at all in the adults. This may indicate that glycocalyx formation and turnover at the tegumental surface is most active during the migration in the host. Once the worms are established in the lung and surrounded by the cyst wall, secretion of the antigen may decrease or stop. Ohara *et al.* (1985) reported that levels of serum IgG antibody against trematode tegument and gut from rats infected with *P. ohirai* reached a peak at 8 weeks postinfection and then declined. This decline seems to correspond to the diminished labeling of the tegumental glycocalyx that we observed in adult worms. Hanna (1980b) pointed out that once juvenile *F. hepatica* enter the bile ducts after migration in the host, antigenic products no longer stimulate the immune system of the host. He also noted that "glycocalyx turnover may help protect the pre-bile duct flukes against immunological attack". Glycocalyx turnover has been similarly reported in *Schistosoma mansoni* (Kusel *et al.*, 1975; Wilson and Barnes, 1977).

The tegumental antigen recognized by the monoclonal antibody was immunobiochemically characterized. The antigen was a very large molecular weight material composed of carbohydrate with antigenic determinant and protein. This would indicate that the antigen was composed of high molecular weight glycoproteins or proteoglycans.

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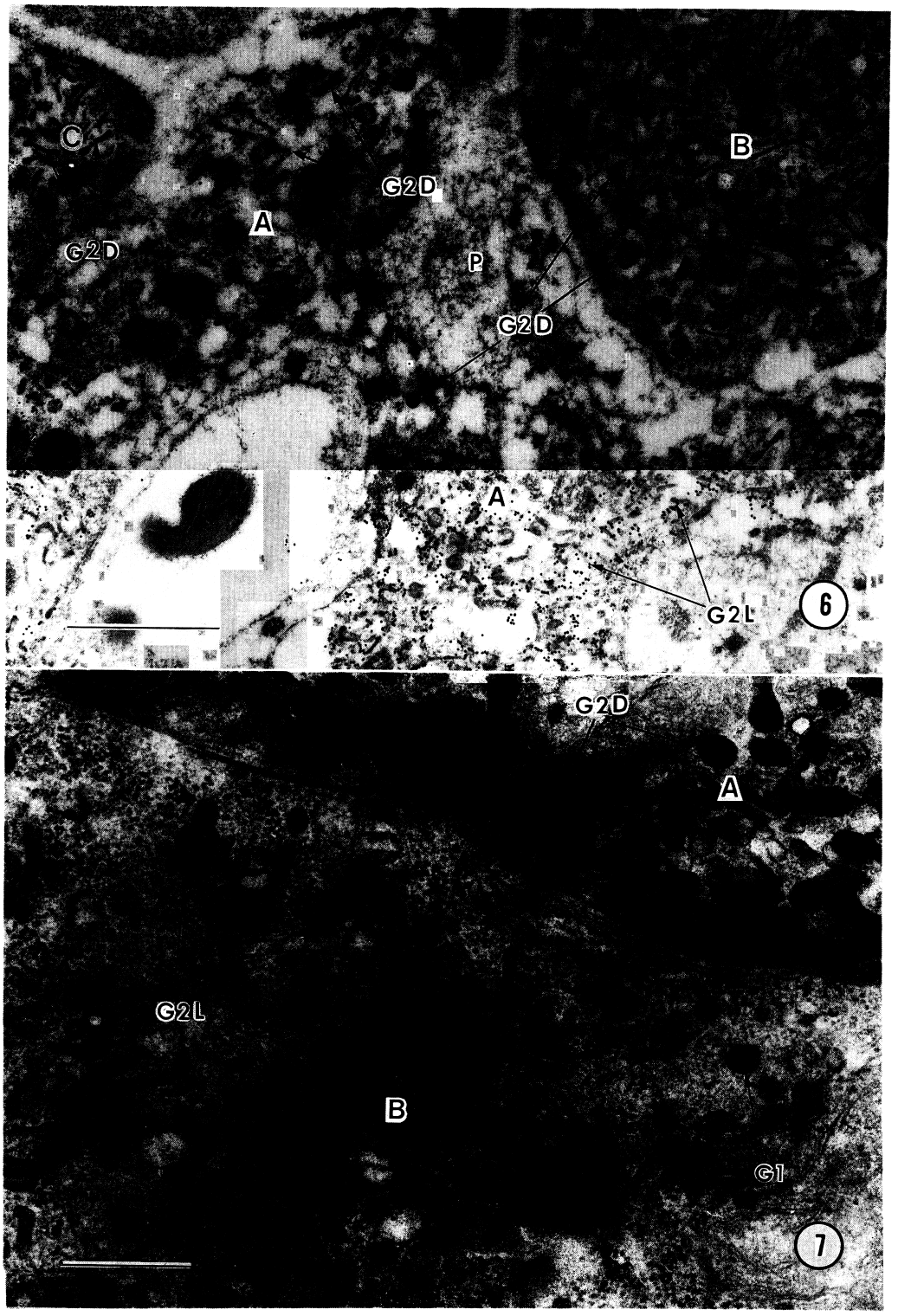
Fig. 6. Tegumental cells of a 50-day-old worm. Almost all G2L granules in cell A are labeled, but G2D granules of cells A, B and C are not labeled. Bar = 1 μm.

Fig. 7. Normal TEM micrograph of the tegumental cells of a 50-day-old worm. Cell A has dense granules (G2D) and cell B bears loosely packed disk-shaped granules (G2L). Bar = 0.5 μm.


C
B
G2D
A
G2D
P
G2D
A
G2L
6
G2D
A
G2L
B
G1
7

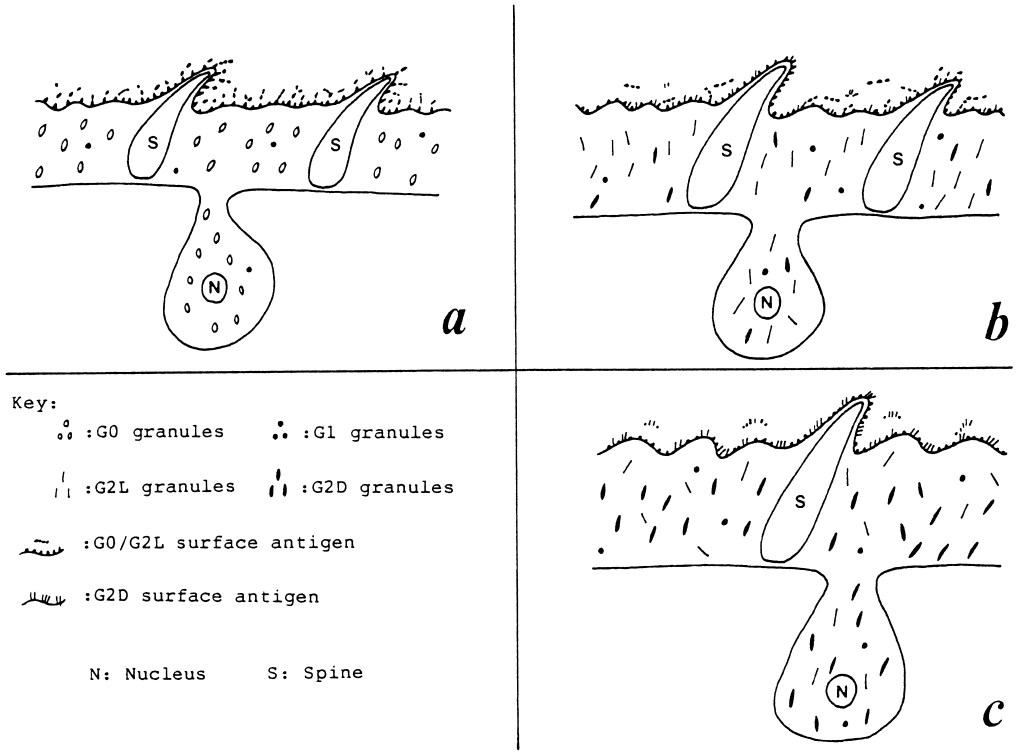


Fig. 8. Diagram summarizing antigenic changes in the tegument and tegumental cells of *P. ohirai* during the development. a: Metacercaria; b: 15-day-old juvenile; c: 50-day-old adult worm.

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