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Ultrastructural Antigenic Localization in the Tissues of *Echinostoma hortense* Observed by Immunogold Labeling Method

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Abstract: This study involved applying rat sera (control, infected and immunized) to adult worm tissue in order to measure the antigenic response. A serum was obtained from rats infected with *E. hortense* metacercaria for 4 weeks. Another immunized serum was taken from rats given a muscle injection with crude adult worm antigen. The detection of antigenic response in *E. hortense* tissue was made by immunogold labeling method and measured through gold particles impregnated in the tissue. The antigenic sites, those with the highest density of gold particles, were the tegmental syncytium, vitelline cells, seminal receptacle and cecum.

Key Words: *Echinostoma hortense*, Immunogold labeling, Antigenic localization, Ultrastructure

INTRODUCTION

The great deal of research on the detection of immune antibodies in final hosts infected by trematodes[16,17,19,20]. Recently in Korea, there has been a report on antigenicity from fluke tissue using the immunogold labeling method. Kwon *et al.*[10] reported that there was a strong immune response in the tegmental syncytium, cytoplasm of the tegmental cell and secretory granule of a premature *Paragonimus westermani* adult worm by IgG obtained from a *P. westermani* infected dog.

Kim *et al.*[9] reported that the strongest immune response in *Clonorchis sinensis* was observed in the excretory secretory antigen by IgG of a *C. sinensis* infected cat. Ahn *et al.*[1] has found that immune response was strong in the tegmental syncytium, tegmental cell and vitelline follicle as a result of the response of cat IgG to *Metagonimus yokogawai*. In addition, antibodies were detected in some of the cecal content.

Since 1980, *E. hortense* has been a frequently found fluke in Korea[13] However there has been a lack of research and report on the immunologic and molecular biologic aspects of this organism. *E. hortense* infection differs from that of *C. sinensis* and *P. westermani* parasites which live in the intestine, and compared with *M. yokogawai* or *Neodiplostonum*

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seoulensis, it the size, sexual organs and metabolism of the adult worms appear to be different. A different antigenic distribution is also observed[12,13,14].

The purpose of this research is to verify antigenic distribution and the origin of antigenicity in the *E. hortense* adult worm by using the immunogold labeling method.

MATERIALS AND METHODS

1. Tissue antigen of *E. hortense*

E. hortense adults were pre-fixed for 2 hours in 1% paraformaldehyde (Sigma, St. Louis, MO, USA) - 0.2% glutaraldehyde (Merck, Darmatadt, F.R. Germany) pH 7.4) and washed in 0.12 M phosphate buffer (pH 7.4). The pre-fixed worms were treated with LR-White regin (Taab, uk) and embedded for 5 days at 40℃. The embedded worms were sectioned by ultrami-crotome for use as a tissue antigen.

2. Crude antigen of *E. hortense*

Four-week old worms were homogenized, ultrasonicated three times for 5～10 sec and centrifuged for 30 min at 4℃, 10,000 rpm. The supernatant was dialyzed with 0.001 M, and 0.005 M Tris buffer (pH 7.4). These specifications were determined by the Bradford method. The resulting substances was used as crude antigen (1 mg/ml).

3. Rat serum

Infected rat sera were obtained from rats which died in 4 weeks after oral infection (OIS). Immunized rat sera were obtained from rats immunized with the *E. hortense* crude antigen (CIS).

4. Immunogold labeling

Immunogold labelling was performed by the method described by Aikawa *et al.* (1990)[2] and Guagliardi *et al.* (1991)[8]. A tissue section of *E. hortense* was attached to a nickel grid and then washed two times in 0.01% Tween 20 (BBL, Cockeysville, USA) and 1% BSA/0.1 M phosphate buffer (PGB). The rat sera (diluted to 1:1,500 for 2 hours at 37℃, and washed in PGB) was applyed to the tissue. The tissue was then treated with gold conjugated bovine serum albumin (Sigma, St. Louis, MO, USA) and poly gold (10 nm) conjugated goat anti-rat IgG (Sigma, St. Louis, MO, USA) for one hour at room temperature, washed in PGB, and fixed in 0.2% glutaraldehyde for 10 min. The immunogold labeled tissue was then washed in 0.1 M PBS and distilled water, dried, double-stained with 6% uranyl acetate (Merk, Darmatadt, F.R. Germany) and lead citrate (Sigma, St. Louis, MO, USA) and observed with electron microscopy (JEM-1200EX II JEOL, Japan). The immune response was determined by the number of gold particles per 0.1 μm^2 of the tissue.

RESULTS

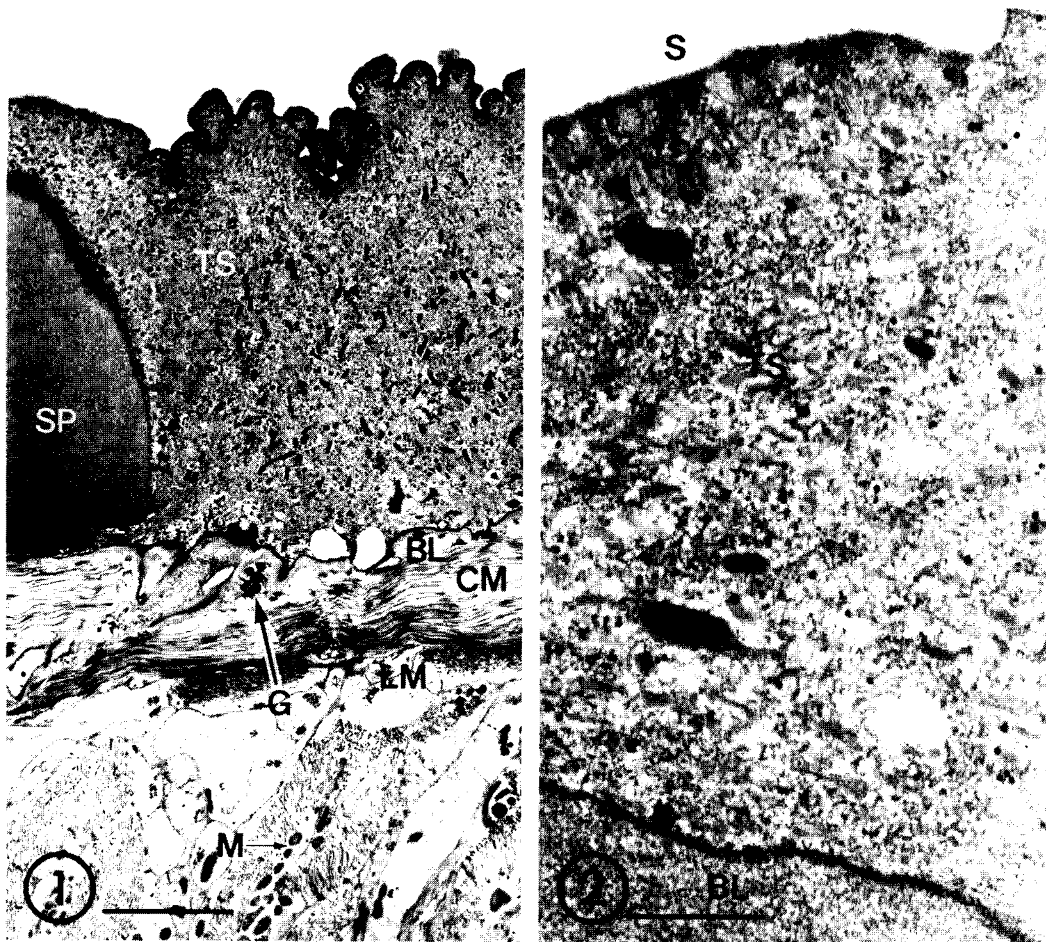
1. *E. hortense* tissue antigen treated with control rat sera

The cytoplasm of the tegumental syncytium had sporadic mitochondria of various sizes with a faint cristae, and densely distributed rod-shaped or elliptical secretory granules. Some vacuoles with low electron density were observed. Large spine rooted out from the cytoplasm through tegument syncytium to the outer side, and the surrounding of the spine showed higher electron density (Fig. 1).

The basal lamina was seen as a uniform line separating the tegumental syncytium and muscle layer. It was 0.4 µm wide and showed low electron density (Fig. 1).

The muscle layer was adjacent to the basal lamina. It was composed of circular muscle, longitudinal muscle and an interstitial matrix of low electron density. It had rod-shaped mitochondria (Fig. 1).

The tegumental cell nuclei had well-developed karyosomes and exhibited a very high electron density. Some cells had rough endo-



Figs. 1-2. Electron micrographs of the *Echinostoma hortense* tegument.

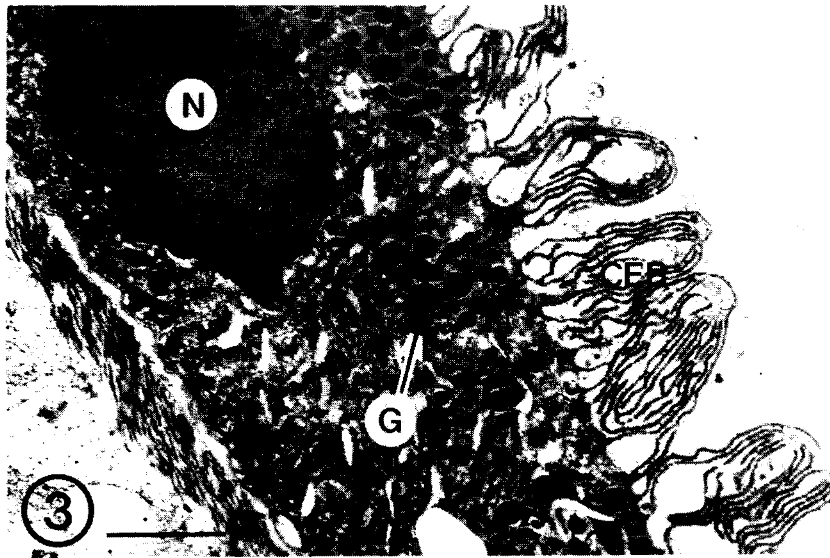
Fig. 1. The tegument of the worm which reacted with rat IgG from the noninfected control serum, showed the tegumental syncytium (TS), spine (SP), basal layer (BL), circular muscle (CM) layer, longitudinal muscle (LM) layer, secretory granules (G) and mitochondria (M). The gold particles were not labeled on the tegument or other portions of the tissue. Bar = 2 μm (X 3,000).

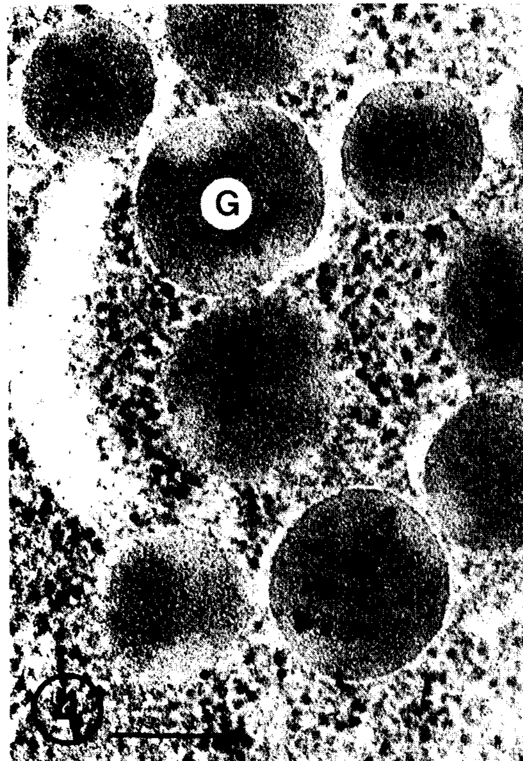
Fig. 2. The tegumental tissue of the worm reacted with the specific antibody from the infected rat. Gold particles were specifically labeled in the tegmental syncyticum. The tegumental syncyticum, surface of worm (S) and basal layer are displayed. Bar = 200 nm (X 30,000).

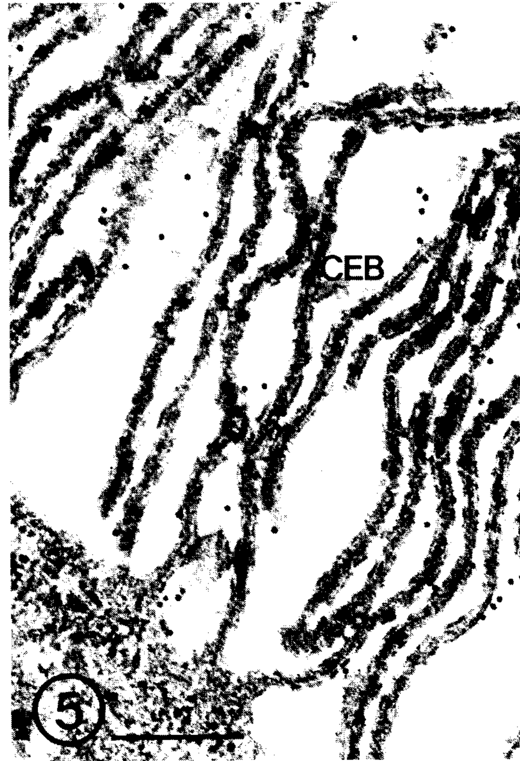
plasmic reticula, mitochondria and golgi complex. The cytoplasm had rod-shaped granules of high electron density (Fig. 1).

The cecum consisted of epithelial lamellae and cecum epithelial lamellae, a membrane structure. The latter developed from epithelial lamellae to lumens, and had secretory granules and digested materials of various sizes and electron density. The epithelial lamellae had tegumental syncytium, circular muscle, epithelial lamellae and some nucleated cells. The nuclei of the nucleated cells had karyosomes of high electron density and peripheral chromatin. Basal lamina existed between the tegumental syncytium and muscle layer. Some interstitial matrix was observed. The cytoplasm was partioned into the three following sections by the cytoplasmic membrane: low electron density, granule-containing, and mitochondria-dense.

The *E. hortense* choroid tissue had various





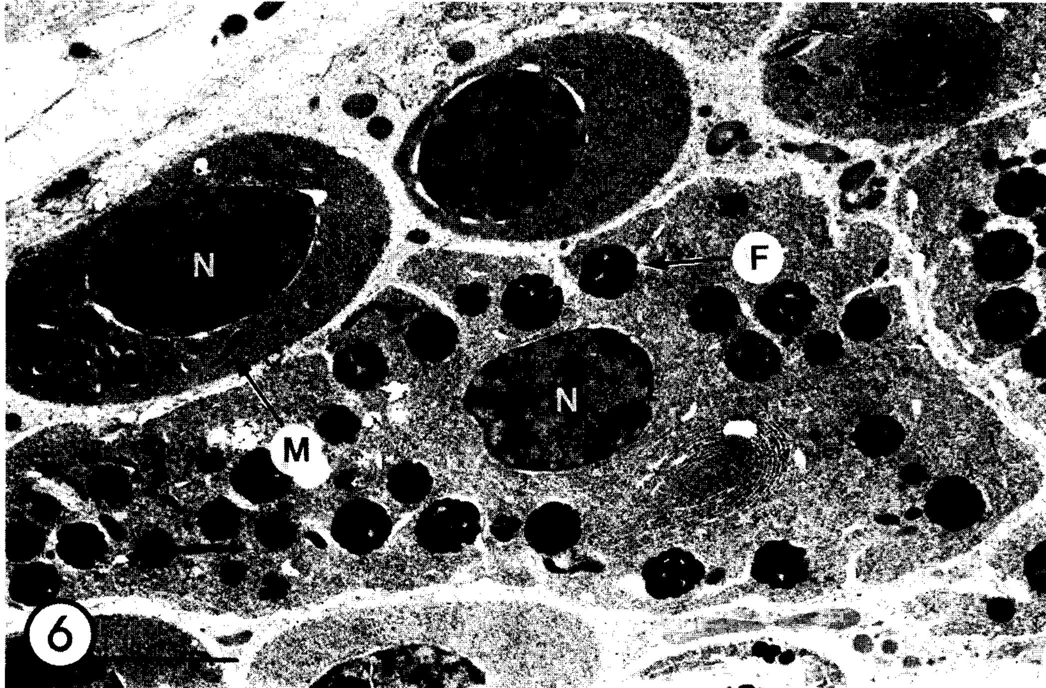


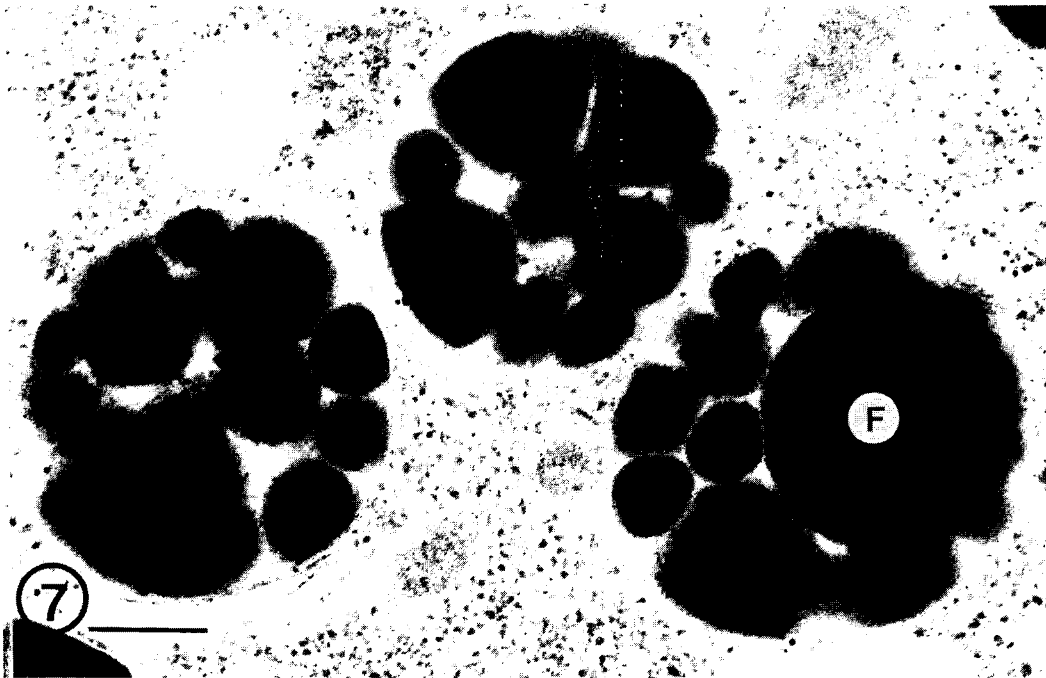
Figs. 3-5. Electron micrograph of the caeca of the worm.

Fig. 3. The caeca of the worm which reacted to sera from rats at 4 weeks after infection. Gold particles were specifically labeled in the secretory granules (G) of cytoplasm and epithelial lamellae in the caeca (CEB). Bar = 1 μm (X 6,000).

Fig. 4. The caeca of the worm which trated with infected rat sera. Gold particles were specifically labeled on the secretory granules. Bar = 200 nm (X 30,000).

Fig. 5. The caeca of the worm which teacted with infected rat sera. Gold particles were specifically labeled on the epithelial lamella. Bar = 100 nm (X 50,000).

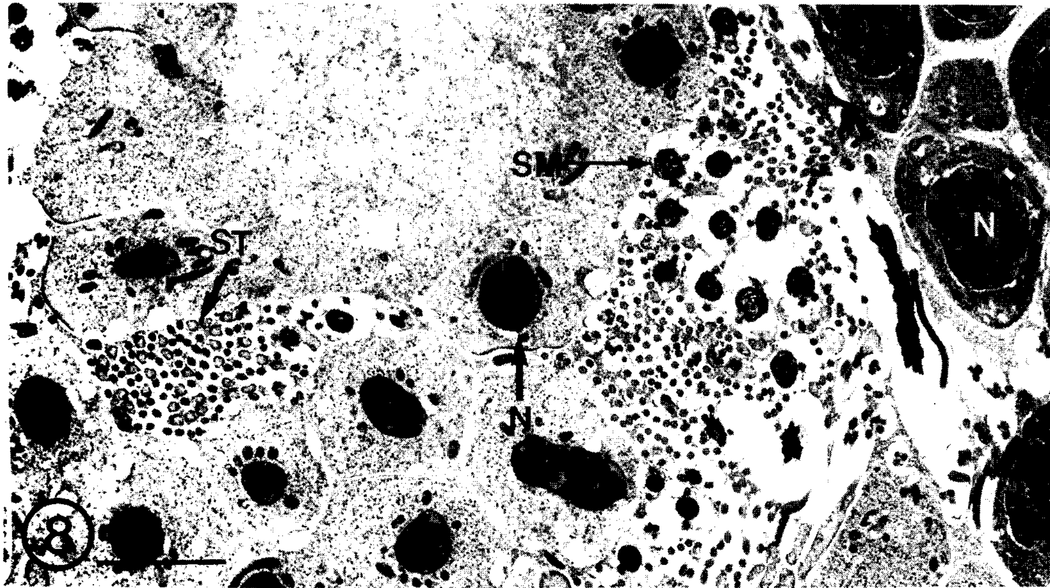


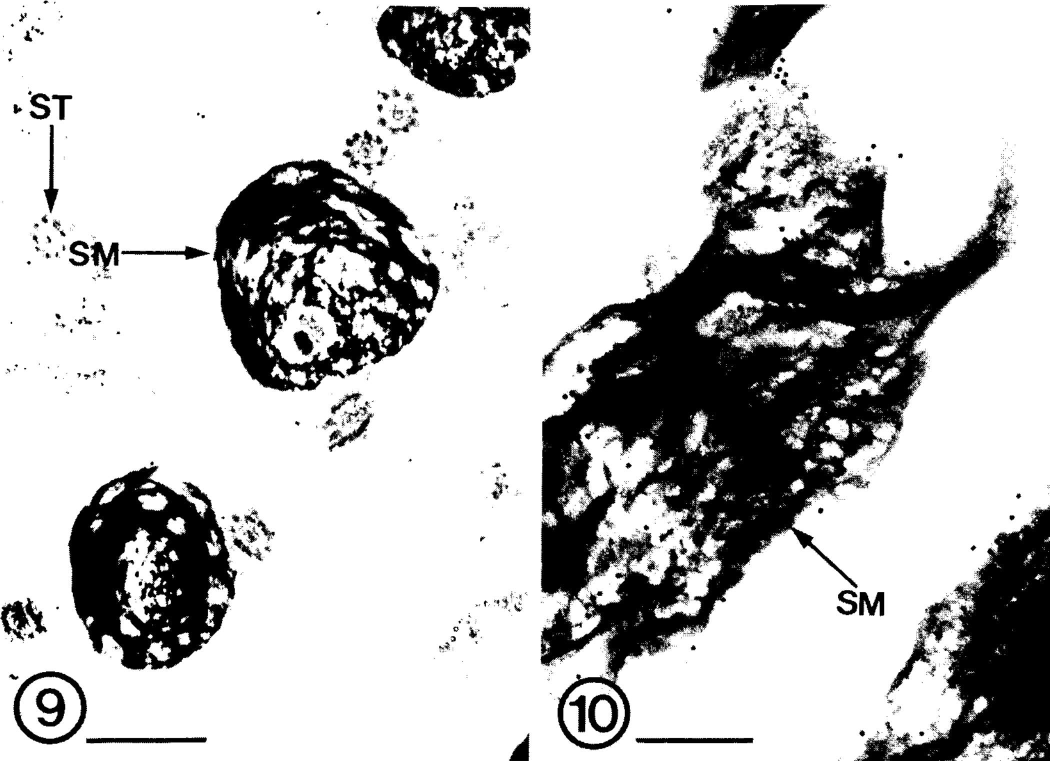


Figs. 6-7. Electron micrograph of the granules (F) in the vitelline follicles of the worm.

Fig. 6. The vitelline follicles of the worm which teacted with infected rat sera. Bar = 2 μm (X 4,000).

Fig. 7. The vitelline folicles of the worm which teacted with infected rat sera. Gold particles were specifically labeled in the granules of vitelline follicles. Bar = 200 nm (X 30,000).





Figs. 8-10. Electron micrograph of the spermatozoa in the seminal receptacle of the worm.

Fig. 8. The spermatozoa (SM) in the seminal receptacle of the worm which teacted with infected sera. Bar = 2 μm (X 2,500).

Fig. 9. The lateral side of spermatozoa (SM) and tail segment of spermatozoa (ST) in the seminal receptacle of the worm which teacted with infected rat sera. Gold particles were specifically labeled in the spermatozoa. Bar = 500 nm (X 15,000).

Fig. 10. The part of longitudinal side of spermatozoa (SM) in the seminal receptacle of the worm which teacted with infected rat sera. Gold particles were specifically labeled in the spermatozoa. Bar = 200 nm (X 30,000).

sizes of secretory granules showing high electron density. Around the secretory granules, rough endoplasmic reticula were observed clearly. Granules near the nuclei of secretory cells were larger.

The vitelline gland had cells containing vesicles composed of high electron density granules. Many nucleated cells surrounded this glard. Multi-layered, annual ring-shaped endoplasmic reticula were observed. Some vitelline cells lacked a nuclei. Most vitelline cells had high electron density and glycogen.

The head of, spermatozoa in the seminal receptacle showed of high electron density while the tail had a relatively low electron density, with well-developed microtubules. There were many nucleated cells densely distributed, composed of electron dense material. The seminal receptacle building blocks also showed high electron density.

2. *E. hortense* tissue antigen treated with infected or immunized rat sera

The tegumental syncytium showed 13±1 gold particles per 0.1 μm^2 with infected rat sera (OIS) and 25±2 with immunized rat sera (CIS) (Table 1). There fore, the tegumental syncytium was thought to have strong antigenicity (25±2). Tegumental syncytium and vacuoles showed an especially strong response. No gold particle labeling was observed in either the mitochondria or the granules (Fig. 2).

No gold particles were observed in basal lamina or muscle layer. But some labeling ocurred in the cytoplasmic process which was connected to the tegumental cell and containing secretory granules. Even though, gold particles were found in the interstitial matrix. However, an immune response was detected in the cytoplasmic material and secretory granules in the cytoplasm (Fig. 2).

The microvilli and lumen of the caecum epithelial structure showed 10±1 gold particles per 0.1 μm^2 with OIS and 50±3 with CIS. The secretory granules of the epithelial lamellae exhibited 10±1 gold particles per 0.1 μm^2 with OIS and 50±1 with CIS, indicating a strong immune response (Fig. 3, 4, 5).

The vitelline was composed of oval secretory granules of various sizes, vitelline follicles and nucleated cells, and had annual ring-shaped endoplasmic reticula. The secretory granules showed 1±1 gold particles per 0.1 μm^2 with OIS and 9±1 with CIS. The cytoplasm, transitional vesicles and endoplasmic reticula of the vitelline follicles, which displayed well-developed rough endoplasmic reticula, all exhibited gold particles (Fig. 6, 7).

A strong immune response was observed in the sperm. The sperm head sections indicated a strong response (8±2 gold particles per 0.1 μm^2 with OIS and 15±3 with CIS). The tail sections showed a weak response (Fig. 8, 9, 10). In addition, the seminal receptacle building

Table 1. Quantitative analysis of immunogold particles in the section of *Echinostoma hortense* with immunized serum and with infected serum

Tissues	Mean number of gold particles / 0.1 μm^2	
	Immunized rat serum (CIS)	Infected rat serum (OIS)
Tegumental syncytium	25±2	13±1
Interstitial martix in parenchyma	8±3	4±2
Secretory granule in vitelline follicle	9±2	1±1
Epithelial lamella and lumen areas in excretory bladder	50±3	10±1
Sperm of seminal receptacle	15±3	8±2

blocks also exhibited a strong immune response.

DISCUSSION

Lee *et al.*[13] reported that the body surface of *E. hortense* was wrinkled transversely and covered with cobble-shaped, pyramidal or rectangular cytoplasmic processes. The tegumental structure was similar to that of *E. revolutum*, but deviated in head spine arrangement and the types and distribution of sensory papillae[6].

The tegument, being a living membrane with physiological and biochemical activity, plays various roles such as nutrient absorption from the host, excretion metabolism and secretion of substances for protection against host digestive enzymes[15].

In this experiment, the *E. hortense* tissue antigen reacted with rat OIS and CIS and was analyzed by immunogold labeling. Strong immune responses were observed in OIS and CIS obtained 4 weeks after infection / immunization. Antibodies from the CIS showed stronger gold labeling, indicating that *E. hortense* worms have some structures which induce strong immune responses from the host. Many gold particles were observed in tegumental syncytium and vacuoles. A moderate level of gold particles were also observed in the cytoplasmic process connected to the tegumental cell and in the secretory granules in cytoplasm. In summary, the *E. hortense* tissue which most dramatically encourage antibody formation are thought to be the tegumental syncytium, tegumental cell and vacuoles.

Sun and Gibson[17], Jeong *et al.*[11], and Cho *et al.*[3] reported that *C. sinensis* caecum and lumen were filled with blood absorbed from the host and waste, and immunoperoxidase staining showed antigen to host in lumen. Sung *et al.*[17] treated a *C. sinensis*-infected animal IgG with the worm tissue and reported immune response in the caecum lumen and epithelial membrane, suggesting that mixture of digestive enzymes and other fields in the lumen had antigenicity. In other words, the mixture was thought to flow into the biliary duct of host, be absorbed and induce antibody formation. Ahn *et al.*[1] reported a strong immune response of caecum tissue of *M. yokogawai* by immunogold labeling. Lee *et al.*[14] reported antigenicity of caecum epithelial membrane and substance in caecum of *P. westermani* by immunoperoxidase staining. Kwon *et al.*[10] reported antigenicity of *P. westermani* worm tissue by immunogold labeling.

In this experiment, caecum microvilli and lumen also showed strong immune response, stronger with CIS than with OIS. Many secretory granules in the caecum epithelial layer also revealed labeled gold particles. Therefore, *E. hortense* caecum and the materials therein are thought to have antigenicity to the host. Erasmus and Popiel[5] and Fukuda *et al.*[7] reported that vitelline cells of trematodes have plenty of glycogen when immature, have high electron density granules such as vitelline globules when mature, and lose nuclei and become filled with only vitelline globules later in the life cycle. Chu *et al.*[4] described the vitelline cells of *C. sinensis* as having plenty of glycogen granules and vitelline globules showing high electron density and strong immunogold labeling. Vitelline cells of *M. yokogawai* and *N. seoulensis* were reported to have secretory granules which showed strong immune responsc[1,12]. Kwon *et al.*[10] reported a strong immune response of vitelline cell secretory granules in *P. westermani.*

In this experiment, vitelline cell secretory granules displayed a strong response as well. Therefore, *E. hortense* vitelline globule substances are thought to act as antigens. Sun and Gibson[17] reported that the seminal receptacles of trematodes showed fluorescent antibodies. Chu *et al.*[4] described a strong immune response in sperm heads in the seminal receptacle of *C. sinensis.* Ahn *et al.*[1] reported a weak response in sperm, but a strong response in the egg of *M. yokogawai.* Lee *et al.*[12] suggested that the

seminal receptacle was the structure from which crude antigen originated in *N. seoulensis*.

In this experiment, the *E. hortense* sperm head showed a strong response while the tail produced a weaker reaction. The sperm body structures also responded strongly. Therefore, those structure, particularly the spermatozoa are thought to induce antibody formation.

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=국문초록=

면역황금표식법을 이용한 호르텐스극구흡충의 조직내 항원성 부위에 관한 연구

동남보건대학 임상병리과[1], 연세대학교 보건과학대학 임상병리학과[2],
원주의과대학 기생충학교실[3] 및 전자현미경실[5], 경산대학교 보건학과[4]

조윤경[1] · 양용석[2] · 이규재[3] · 김인식[2] · 류장근[4] · 송미숙[5]

호르텐스극구흡충을 흰쥐에게 경구 감염시키고 4주 후에 성충을 획득하여 고정, 포매 및 절편하여 조직항원 분포 부위를 관찰하였다. 호르텐스극구흡충의 항체는 호르텐스극구흡충 피낭유충을 흰쥐에게 경구 감염시키고 4주 후에 채혈한 혈청과 호르텐스극구흡충 조항원을 면역증강제와 함께 흰쥐에게 근육 주사하여 채혈한 혈청을 이용하였으며, 이 혈청을 반응시켜서 호르텐스극구흡충 조직내의 항원성 분포를 규명하였다. 한편, 면역황금표식법에 의한 미세조직의 항원성 반응 여부는 조직에 표지된 황금입자로 판정하였다. 그 결과 충체 조직에서 황금입자와 강하게 반응하는 미세조직은 표피층의 표지합포체, 난황세포의 구성물질, 유연조직의 분비과립 그리고 배설낭의 구성물질 등에서 반응이 강하게 나타났다. 이상의 결과를 볼 때 호르텐스극구흡충의 항원은 생식세포와 소화분비 및 소화관계통에서 분비 유래되는 것으로 분석되었다.

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