

## Localization of worm antigen in *Neodiplostomum seoulense* by immuno-electronmicroscopy

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**Abstract:** The localization of worm antigen of *Neodiplostomum seoulense* was examined by immuno-electronmicroscopic observation. Not only the immunized serum of mice with crude worm extract of *N. seoulense* but also serum of infected mouse were reacted to the worm section. Using immunized serum as primary antibody, the gold particles were deposited on the rough endoplasmic reticulum of the cell of tribocytic organ, spermatozoa in the seminal vesicle, microvilli of the caecum and vitelline follicle. Using infected serum, gold particles were deposited only on the vitelline follicle prominently. This finding suggested that the tribocytic organ, seminal vesicle, caeca and vitelline follicles may play a role of antigen to immunized serum with crude worm extract of *N. seoulense*, whereas the vitelline follicle, to the infected serum.

**Key words:** *Neodiplostomum seoulense*, immuno-electronmicroscopy, antigen, follicle, immunization, mouse

### INTRODUCTION

*Neodiplostomum seoulense* (= *Fibricola seoulensis*) infection is one of the endemic intestinal trematodiasis in Korea (Seo, 1990; Hong and Shoop 1995). Till now, 27 human cases have been reported (Huh *et al.*, 1994). Human can be infected with this worm by ingestion of raw snake or frog. The infection can be manifested from severe gastrointestinal symptoms such as abdominal pain or diarrhea to no symptom. This variable manifestation may be explained due to the difference of

worm burden, and to the difference of immune reaction. For the immunological approach to the intestinal trematodiasis, *N. seoulense* is one of the best model due to its typical clinical and histopathological findings (Seo, 1990). In the animal experiment, worm specific IgG or IgA increased 4 weeks post-infection (PI) (Kho, 1992; Huh *et al.*, 1995). Although this kind of immune reaction is not closely related with worm expulsion phenomena, it is important to know which component of worm is strongly antigenic (Kim *et al.*, 1995). It can be used as an explanation of the source of immune reaction to host. The antigenic organ may secrete the enzyme for the penetration of the host tissue and it can play a role of immune evasion in host-parasite interrelation.

Kho (1992) suggested that *N. seoulense*-specific IgG of rat was originated from the seminal receptacle and testis of the worm by immunohistochemical method. However, there

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was no datum using *N. seoulense*-specific IgG of mouse. Also there might be some difference between two kinds of serum i.e. immunized serum with worm extract and serum of infected animals. We tried to observe the localization of the worm antigen in *N. seoulense* by immuno-electronmicroscopy to know the origin of strong antigenicity from the worm. It may elucidate which organ is antigenic to immunized and infected serum of mouse.

## MATERIALS AND METHODS

### Recovery of the worm

Metacercariae were collected from the European grass snake (*Rhabdophis tigrina*) by artificial digestion in 0.6% pepsin in 1% HCl at 37°C for 2 hours. Five mice (BALB/c, 4 weeks-old, regardless of sex) were infected with 300 metacercariae and recovered from small intestine 2 weeks PI. Another five mice were infected with 300 metacercariae and survived ones were sacrificed 4 weeks PI for the serum collection.

### Primary antibody and conjugate

Recovered worms were homogenized and centrifuged at 15,000 g for 3 hours. Supernatant was used as the crude worm extract antigen for the immunization. Crude worm extract was diluted to 0.5 mg/ml in 0.85% NaCl. Worm antigen was mixed with Freund's conjugate (complete) with a ratio of 1:1, and 0.3 ml was injected to mouse (BALB/c, 4 weeks-old) femoral muscle. Two weeks later, 0.2 ml of antigen was injected again to femoral muscle. Two weeks later, 0.1 ml of antigen was injected to the tail vein and blood was collected by cervical capitation three days later of final injection. Serum obtained after centrifuge was kept at -70°C until use. Serum of the infected mouse and that of uninfected mouse were collected 4 weeks PI and kept -70°C until use. Conjugate was the 15 nm gold labeled goat anti-mouse IgG (Fc) (Amersham International).

### Immuno-electronmicroscopic method

Worms were fixed in 1% paraformaldehyde, 0.2% glutaraldehyde in 0.1 M phosphate

buffer (PB, pH 7.4) for 2 hours at 4°C. Samples were dehydrated in a graded series of ethanol at 4°C and embedded in Lowcryl HM20. The resin was polymerized for a day. Thin section (60 nm) was cut and mounted on 300 mesh nickel grid. Grid was incubated with drops of blocking solution (0.9% NaCl, 5% skim milk, 0.01% (v/v) Tween 20 in 0.1 M PB) for 15 minutes. Each grid was incubated with 1:50 dilution of primary antibody in blocking solution for 2 hours at room temperature. It was washed thoroughly in the solution of PBS-BSA-Tween (0.9% NaCl, 1% bovine serum albumin (fraction V), 0.01% (v/v) Tween 20 in 0.1 M PB). It was incubated with 1:20 dilution of conjugate in PBS-BSA-Tween at room temperature for 1 hour and washed in PB-Tween (0.01% (v/v) Tween 20 in 0.1 M PB) with 3 changes for 10 min. After washing with distilled water, it was dried and stained with 2% (w/v) uranyl acetate in 50% methanol and Reynolds lead citrate. Stained section was examined with Zeiss transmission electron-microscope (100 SX). Gold particles in the area of 0.1  $\mu\text{m}^2$  were counted in each structure for 10 areas.

## RESULTS

Thin section of *N. seoulense* treated with the immunized serum showed high concentration of antibody over the rough endoplasmic reticulum (rER) of the cell of tribocytic organ, spermatozoa in the seminal vesicle, microvilli of the caecum, and vitelline follicle. Using infected serum, gold particles were deposited mainly on the vitelline follicle and a few gold particles were deposited on microvilli of caecum. The quantitative analysis of immunogold labelling was shown in Table 1.

In the tegument, there were a few gold particle on the rER. On the spine, basal layer, longitudinal muscle, circular muscle, granule, and nucleus and mitochondria of the subtegumental cells, no reactions were observed. This finding was similar using both immunized serum and infected serum (Figs. 1 & 2). In the tribocytic organ, the rER beneath the cell of outer surface showed the deposition of the gold particles using immunized serum ( $13 \pm 2$  particles/0.1  $\mu\text{m}^2$ ). However, the

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

Tissues	Mean number of gold particles/0.1 $\mu\text{m}^2$	
	immunized mouse IgG	Infected mouse IgG
tegument	1 $\pm$ 1	1 $\pm$ 1
rER of the tribocytic organ	13 $\pm$ 2	2 $\pm$ 1
interstitial matrix between spermatozoa	17 $\pm$ 3	2 $\pm$ 1
space surrounding sperm head	31 $\pm$ 3	2 $\pm$ 1
microvilli of the caeca	28 $\pm$ 2	4 $\pm$ 2
interstitial area between and surrounding the granules of the vitelline follicle	34 $\pm$ 3	16 $\pm$ 3

reaction was weak using infected serum ( $2 \pm 1/0.1 \mu\text{m}^2$ ). On the granules and mitochondria, there was no reaction (Figs. 3, 4 & 5). On the interstitial matrix between spermatozoa in the seminal vesicle, the gold particle existed. Also on the space surrounding sperm head, there were gold particles. This is remarkable when using immunized serum ( $31 \pm 3/0.1 \mu\text{m}^2$ ) (Figs. 6, 7 & 8). On the microvilli of the caeca, there were many gold particles using immunized serum ( $28 \pm 2/0.1 \mu\text{m}^2$ ) (Fig. 9). Also the granules were reactive. The number decreased in the section reacted with infected serum ( $4 \pm 2/0.1 \mu\text{m}^2$ ) (Fig. 10). On the interstitial area between and surrounding the granules of the vitelline follicle, there were many gold particles using immunized ( $34 \pm 4/0.1 \mu\text{m}^2$ ) and infected serum ( $16 \pm 3/0.1 \mu\text{m}^2$ ) (Figs. 11 & 12). Usually vitelline gland contains 3-4 vitelline follicular cells that possess some glycogen-containing granules seen as electron dense round granule. The reaction findings using uninfected serum showed no gold particle in the worm (Data not shown).

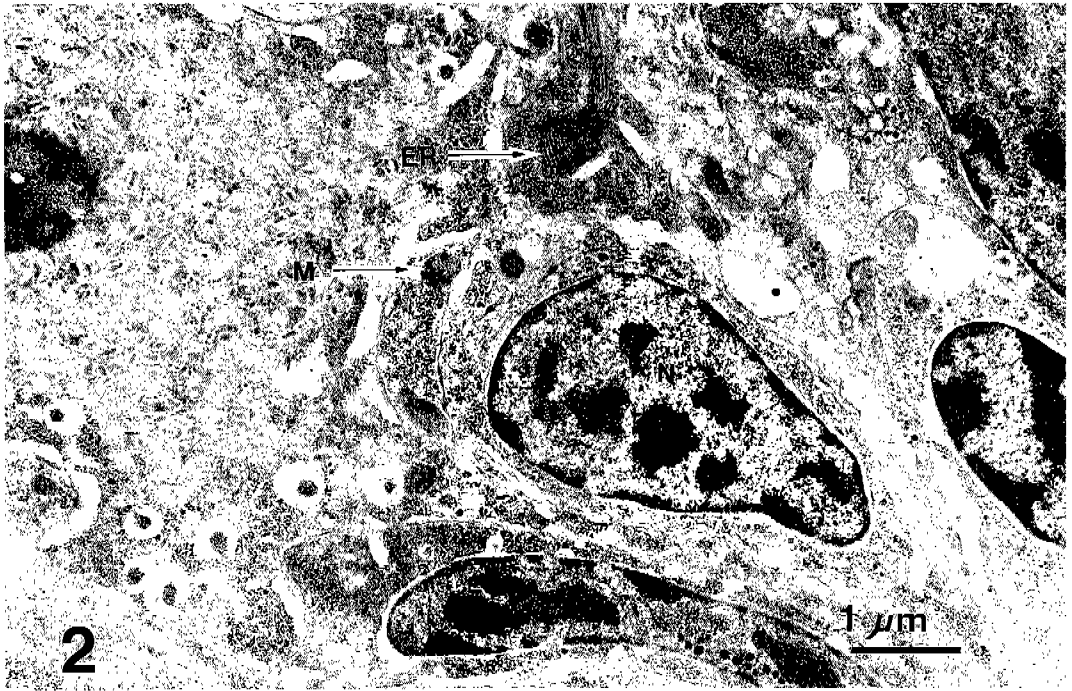
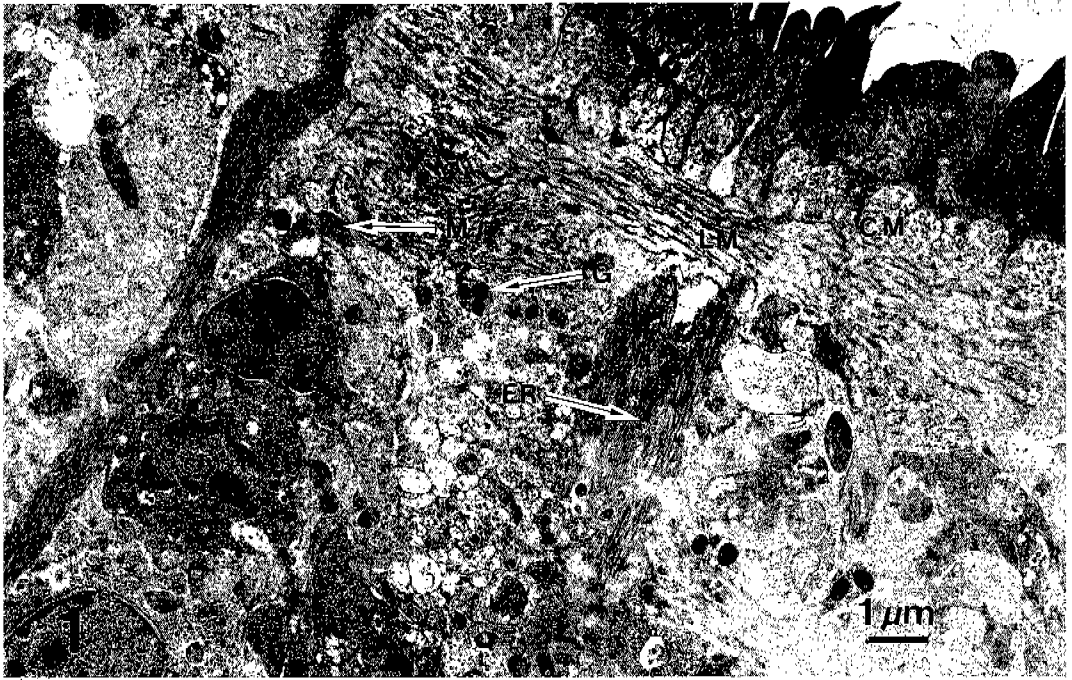
## DISCUSSION

Above results told us that the rough endoplasmic reticulum (rER) of the cell of tribocytic organ, spermatozoa in the seminal vesicle, microvilli of the caecum, and vitelline follicle were strong antigen for the immunized serum. Tegumental cytoplasmic layer, basal layer, tegumental cell, muscles, interstitial matrix of parenchyme did not show strong antigenicity. This finding is somewhat different that of *Clonorchis sinensis* (Chu *et al.*, 1990).

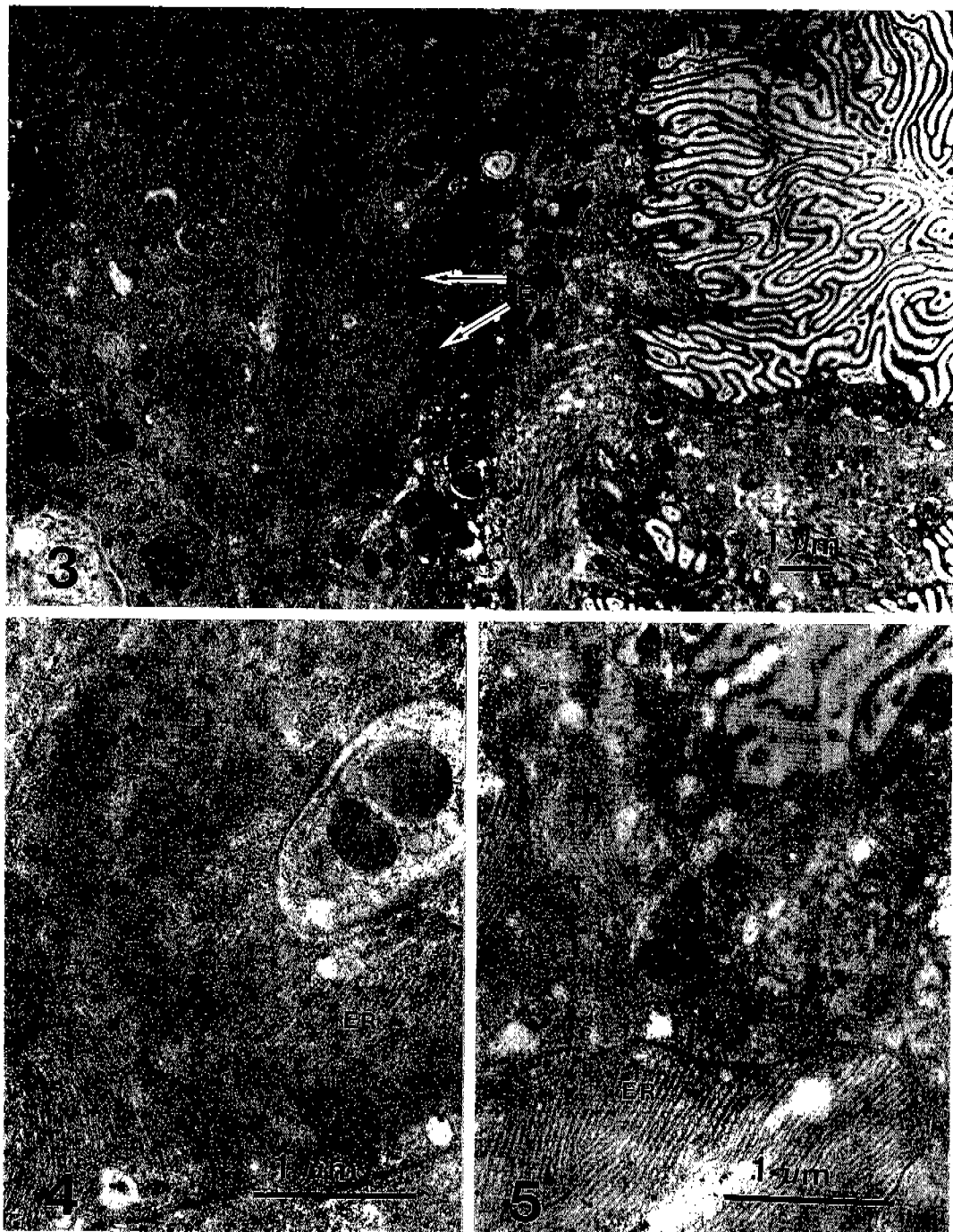
In the *C. sinensis*, the immunized rabbit IgG reacted at basal layer (basal lamina), interstitial matrix of parenchyme, although the microvilli (epithelial lamella) of caeca, and vitelline gland are antigenic like in *N. seoulense*. In *Fasciola sp.*, the cysteine protease were consistently deposited on the electron dense secretory granule and intestinal epithelial cells and on the intestinal contents, which suggested that the granules may play a role of degradation of host tissues (Yamasaki *et al.*, 1992). In the *N. seoulense*, there is also a possibility of localization of cysteine protease in the microvilli or granules of the caeca, which should be pursued later.

The reactive sites with immunized serum are all active metabolic sites. The rER contain the ribosome which is site of protein synthesis. The sperm is believed to secrete the metabolite or enzyme which is antigenic to immunized serum. Microvilli of the caeca is intimate organ with host tissue. The secretions from ingested host tissue can do a role of antigenic to host immunized serum. Vitelline follicle secreted the materials which contribute to the egg formation. The common findings of above antigenic tissues or organ is that they are secretory in function. During the preparation of the worm antigen, the secrete was melted in the supernatant. They can be antigenic to immunized serum.

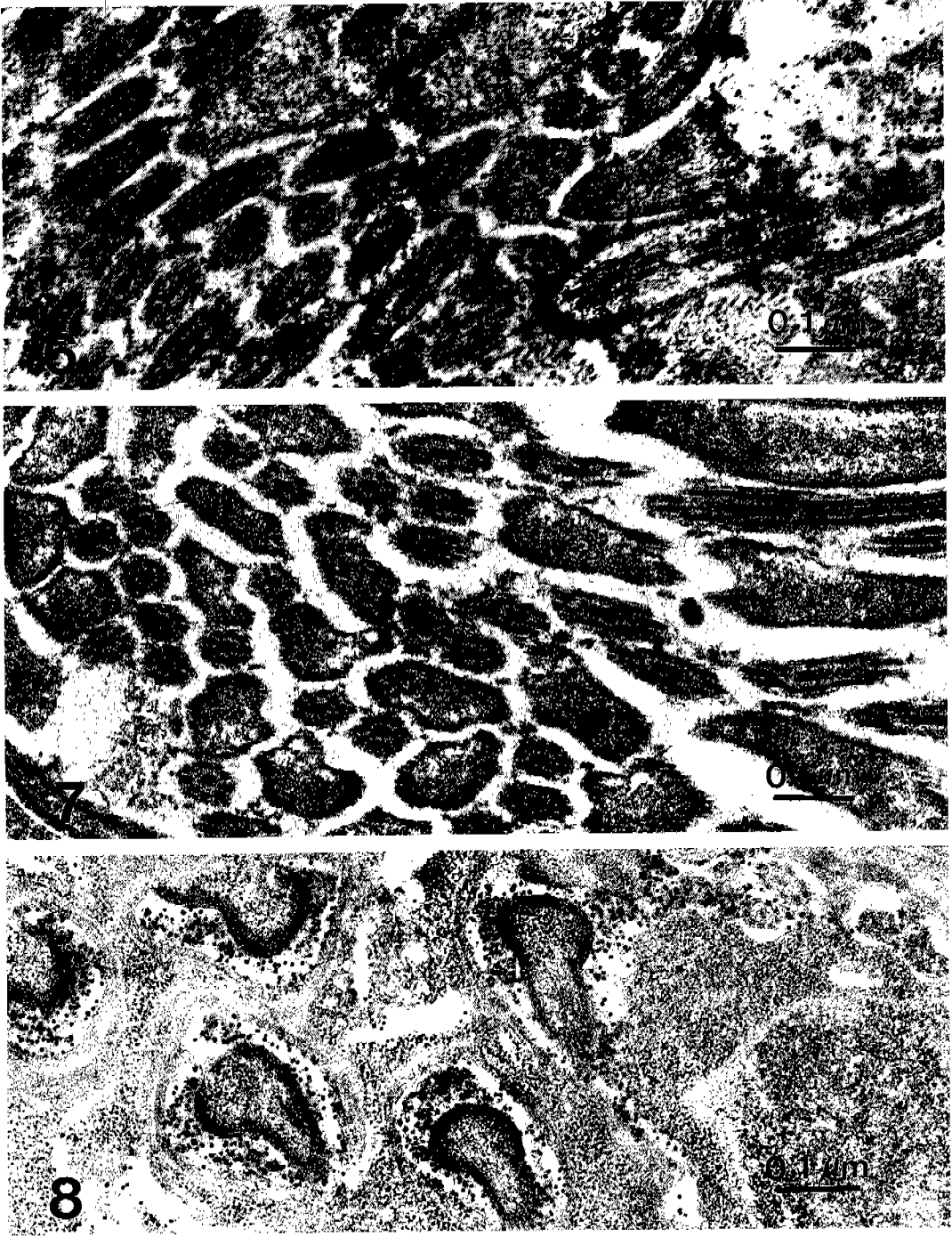
The finding using infected serum was different with that of Kho (1992). He mentioned that the strong antigenicity is shown in the seminal vesicle, testes, spermatic canal and weak antigenicity in the vitelline follicle. In this experiment only weak reaction on spermatozoa in the seminal vesicle was



**Fig. 1.** Tegument of *Neodiplostomum seoulense* treated with immunized serum. A few number of gold particles were deposited on the rough endoplasmic reticulum (rER). S: spine, CM: circular muscle. LM: longitudinal muscle. G: granule, M: mitochondria, N: nucleus. **Fig. 2.** Tegument of *N. seoulense* treated with infected serum. A very few number of gold particles were deposited on the rER. M: mitochondria, N: nucleus.

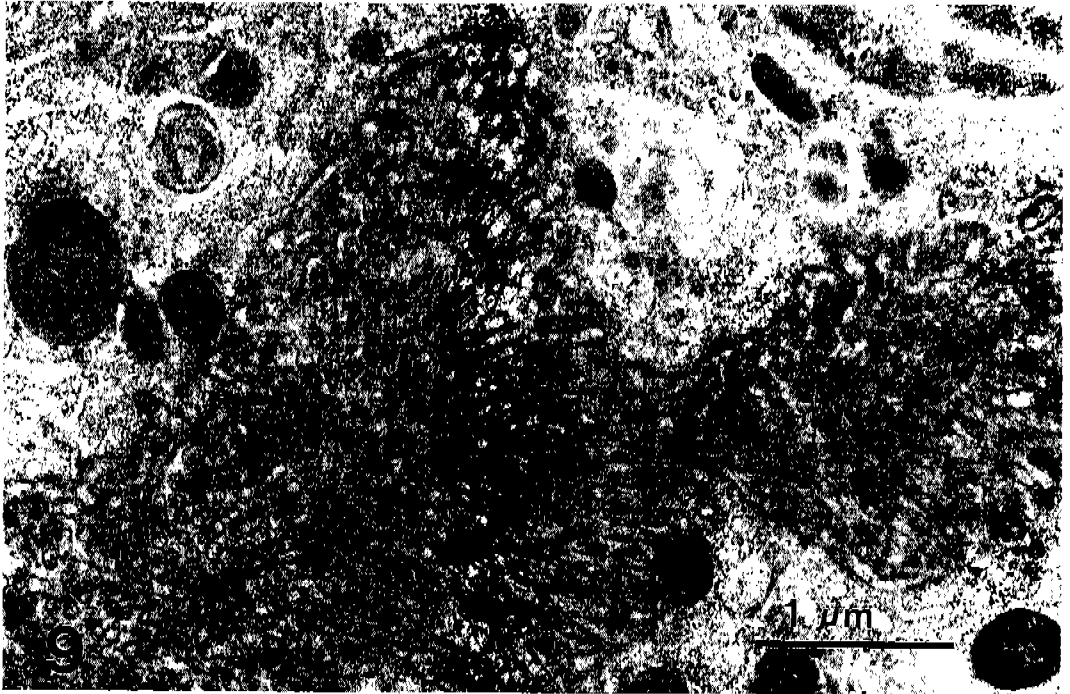


**Fig. 3.** Tribocytic organ of *N. seoulense* treated with immunized serum. Gold particles ( $13 \pm 2$  particles/ $0.1 \mu\text{m}^2$ ) were deposited on the rough endoplasmic reticulum (ER). G: granule. **Fig. 4.** Ibid. High magnification. **Fig. 5.** Tribocytic organ of *N. seoulense* treated with infected serum. Gold particles ( $2 \pm 1$  particles/ $0.1 \mu\text{m}^2$ ) were deposited on the rER. G: granule.

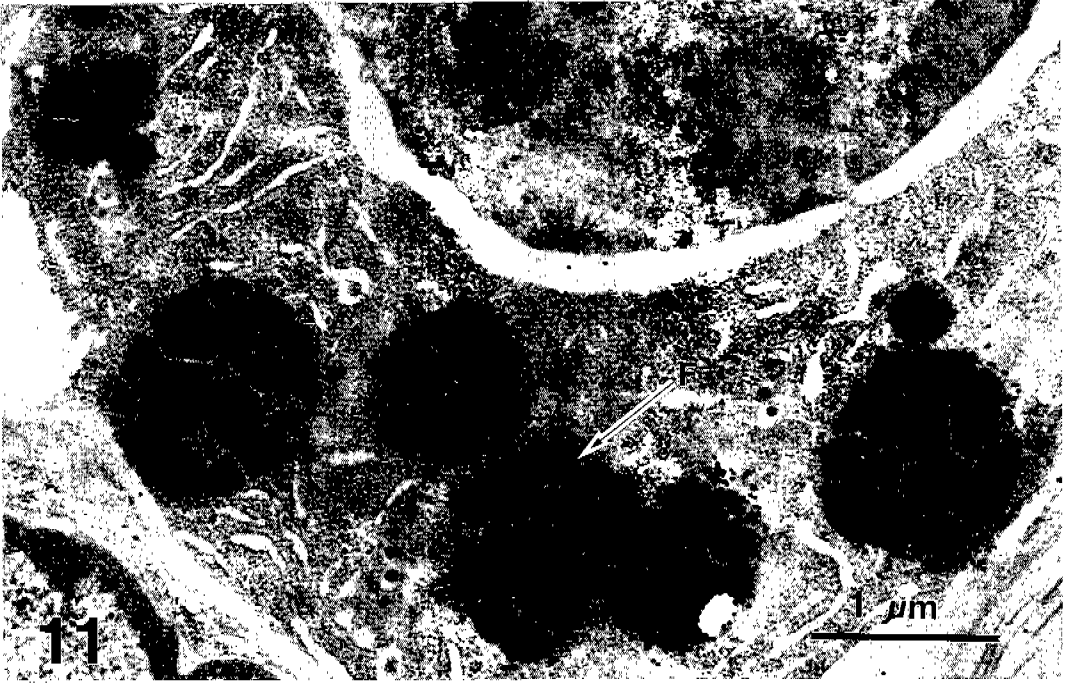


**Fig. 6.** Spermatozoa in the seminal vesicle treated with immunized serum. Gold particles were deposited on the interstitial matrix between spermatozoa. **Fig. 7.** Spermatozoa in the seminal vesicle treated with infected serum. Gold particles were deposited on the interstitial matrix between spermatozoa. **Fig. 8.** Head of spermatozoa in the seminal vesicle treated with immunized serum. Gold particles ( $31 \pm 4$  particles/ $0.1 \mu\text{m}^2$ ) were deposited on the space surrounding sperm head.





**Fig. 9.** Microvilli of the caeca treated with immunized serum. Gold particles ( $28 \pm 2$  particles/ $0.1 \mu\text{m}^2$ ) were deposited on the microvilli (V) of caeca. **Fig. 10.** Microvilli of the caeca treated with infected serum. Gold particles ( $4 \pm 2$  particles/ $0.1 \mu\text{m}^2$ ) were deposited on the microvilli (V) of caeca.



**Fig. 11.** Vitelline follicle treated with immunized serum. Gold particles ( $34 \pm 4$  particles/ $0.1 \mu\text{m}^2$ ) were deposited on the interstitial area between and surrounding the granules of the vitelline follicle (F). **Fig. 12.** Vitelline follicle treated with infected serum. Gold particles ( $16 \pm 3$  particles/ $0.1 \mu\text{m}^2$ ) were deposited on the interstitial area between and surrounding the granules of the vitelline follicle (F).



observed and strong reaction from vitelline follicle. This kind of differences probably originated from the differences of immune reaction of experimental animals and/or the difference of sensitivity of the staining method. Since the excretory-secretory antigen was released during the worm infection, strong excretory-secretory antigen can be said to be originated from vitelline follicle. This phenomenon was also shown in other helminths. When infected serum was used for primary antibody in immuno-electron-microscopic examination, secretory protein from the intestinal caeca or egg-related protein such as vitelline follicle had been known to show strong antigenicity in *Paragonimus westermani* (Kwon *et al.*, 1991; Rim *et al.*, 1992), *C. sinensis* (Chu *et al.*, 1992), *Metagonimus yokogawai* (Ahn *et al.*, 1991) and *Paragonimus iloktsuenensis* (Kim *et al.*, 1995). Secretes from vitelline follicle are believed to be a source of excretory-secretory antigen in neodiplostomiasis.

#### REFERENCES

- Ahn H, Rim HJ, Kim SJ (1991) Antigenic localities in the tissues of *Metagonimus yokogawai* observed by immunogold labeling method. *Korean J Parasitol* **29**: 245-257.
- Chu BD, Rim HJ, Kim SJ (1990) A study on the body fluid antigen of *Clonorchis sinensis* using immunogold labeling method. *Korean J Parasitol* **28**: 11-23.
- Hong ST, Shoop WL (1995) *Neodiplostomum seoulense*, the emended name for *Neodiplostomum seoulensis*. *Korean J Parasitol* **33**: 399.
- Huh S, Lee SU, Huh SC (1994) A follow-up examination of intestinal parasitic infections of the Army soldiers in Whachon-gun, Korea. *Korean J Parasitol* **32**: 61-63.
- Huh S, Lee SU, Won MH, Jeong YG, Kwon YH, Choi CS (1995) IgA response in mice infected with *Neodiplostomum seoulensis*. *Korean J Parasitol* **33**: 55-60.
- Kho WG (1992) Analysis of antigenic protein of *Fibricola seoulensis*. PhD Thesis Dissertation. Seoul National University, Seoul.
- Kim HS, Lee OR (1995) Ultrastructural antigenic localization in *Paragonimus iloktsuenensis* during developmental stage by immunogold labeling method. *Korean J Parasitol* **33**: 365-376.
- Kwon OS, Lee JS, Rim HJ, Kim SJ (1991) Antigenic localities in the tissues of the young adult of *Paragonimus westermani* using immunogold labeling method. *Korean J Parasitol* **29**: 31-41.
- Rim HJ, Kim SJ, Sun IJ, Lee JS (1992) Antigenic localities in the tissues of *Paragonimus westermani* by developing stages using immunogold labeling method. *Korean J Parasitol* **30**: 1-14.
- Seo BS (1990) *Fibricola seoulensis* Seo, Rim and Lee, 1964 (Trematoda) and fibricoliasis in man. *Seoul J Med* **31**: 61-96.
- Yamasaki H, Kominami E, Aoki T (1992) Immunocytochemical localization of a cysteine protease in adult worms of the liver fluke *Fasciola* sp. *Parasitol Res* **78**: 574-580.

=초록=

면역전자현미경법으로 관찰한 서울주걱흡충에서 총체 항원의 분포

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서울주걱흡충의 총체 항원의 유래 부위를 알기 위하여 면역전자현미경법으로 관찰하였다. 총체 조항원으로 면역시킨 마우스 혈청을 분리하여 총체 조직과 반응시켰다. 조직 부위 중 조직용해구 세포의 과립세포질세망, 저정낭의 정자 사이 조직과 정자머리 주위, 맹장의 미세음모, 난황이 면역된 마우스 혈청과 강하게 반응하였다. 감염 마우스 혈청과는 난황 과립이 강하게 반응하고, 맹장의 미세음모가 약하게, 다른 부위는 매우 약하게 반응하였다. 그러므로 조직용해구, 저정낭, 맹장, 난황이 조항원의 유래 부위이고, 총체분비물의 항원은 난황에서 유래하는 것이 가장 강하게 작용하는 것으로 생각한다.

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