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Electron Microscopic Studies on Surface Structures of *Angiostrongylus cantonensis* Egg Shells

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Abstract

The egg shells of *Angiostrongylus cantonensis* were observed by means of transmission electron microscope. The eggs used in this study were those recovered from the uterus and those cultured *in vitro* for 1 to 7 days. Immature eggs were found in the middle part of the uterus without any noticeable structures on egg shells. In the lower part, innumerable chitinous projections were observed on egg shells. On the 1st and 3rd days of cultivation, the projections started to have a few microvilli on the tip. Thereafter, these structures decreased in number as the eggs aged. Considering the life cycle of this nematode, these structures found on egg shells seems to play a role in prevent the eggs from being carried away by the blood.

Key words: *Angiostrongylus cantonensis*, egg shell, transmission electron microscope, ultrastructure, nematoda

Introduction

Angiostrongylus cantonensis is essentially a nematode that is parasitic on rats as the final host, lodging in the pulmonary artery. However, as they infect also human beings occasionally, causing tropical eosinophilic meningoencephalitis, they are noted as the causative parasite of zoonoses. Eggs of this nematode usually deposited in the pulmonary artery of the final host, hatch after blocking blood vessels in the lung, and appear in the feces as first stage larvae. Therefore, detailed study of growth at the very early stage of this nematode, from egg to first stage larva, was rather limited.

The authors (Uga *et al.*, 1982, 1984) have reported a series of studies on *in vitro* cultivation of *A. cantonensis*. One of the studies revealed that eggs of this nematode grew well on a condition of *in vitro*, and hatched on the 8th day of cultivation, and became first stage larvae. This culture method makes it possible to obtain numerous eggs which were synchronized in their developmental stage. In the previous paper (Uga and Matsumura, 1982) we reported the presence of some interesting ultrastructures on the surface of egg shells. However, neither detailes of their forms nor structural changes with aging of eggs were not mentioned. The purpose of this study was to clarify the surface structures on *Angiostrongylus cantonensis* egg shells.

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Materials and Methods

Laboratory Maintenance of Angiostrongylus cantonensis

The *A. cantonensis* used in this study were maintained in our laboratory by using the aquatic snail, *Biomphalaria glabrata* and albino rats (*Rattus norvegicus*). About 20 third stage larvae recovered from *B. glabrata* were administered orally to each rat. Forty to forty-five days after oral administration, fecal examination of the rats was performed to check the excretion of the first stage larvae. Only the rats which discharged the first stage larvae in their feces were used. The rats were killed with ether. Immediately after death, the heart and pulmonary artery and its branches were care-

fully dissected and examined for worms.

Preparation and Observation of Eggs

The female worms collected were dissected under the stereomicroscope, and 1 to 2 mm lengths of the middle and terminal end of the uterus were kept to observe under the transmission electron microscope.

For cultivation only the eggs isolated from the terminal end of the uterus were used. The terminal ends of the uteri were placed in Petri dishes with 0.2 to 0.5 ml of culture medium. They were then dissected by forceps and scissors. After leaving them for 10 min, the released eggs were recovered. The eggs were cultured in a medium of NCTC 109 supplemented with rat serum at a concentration of 50%, and used for observations on the 1st, 3rd, 5th and 7th day of cultivation.

To prepare the specimens for electron microscopy, the materials were prefixed for 1.5 hr in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.3) at 4°C, and then postfixed for 1 hr in 2% OsO_4 solution at 4°C. They were dehydrated in a graded series of alcohol and propylene oxide, embedded in Epon, sectioned, and observed with a Hitachi HS-9 electron microscope.

Results

Fig. 1 shows the development of eggs cultured. The eggs recovered from the middle part of the uterus were considered to be infertile as they did not grow *in vitro* at all. The eggs obtained from the terminal end of the uterus were fertile and grew well *in vitro*. They grew to 8 – 16 cells on the 1st day of cultivation, to 32 cells or more on the 3rd day, and to embryonated eggs on the 5th day. At this stage movements of larvae were observable although they were very slow. On the 7th day of cultivation movement of the larvae was more active with egg shells soft and eggs changed in their shapes because of the movements of the larvae. When the eggs thus cultured were administered to intermediate host snails, even the embryonated eggs on the 6th day of cultivation showed infectivity, indicating that these eggs grew normally.

Days after cultivation	Stages of development	Illustration (Fig. No.)
0(middle)*	1	(2)
0(lower)	1	(3,4)
1	8-16	(5,6)
3	32 or more	(7)
5	embryonated	(8,9)
7	embryonated †	(10)

Fig. 1 Change of fine structure on egg shells accompany with the development of *A. cantonensis* eggs *in vitro*. *Eggs were recovered from the middle part of the uterus. †Egg shells changed their shapes because of the movement of the larvae.

Eggs in Middle Part of Uterus

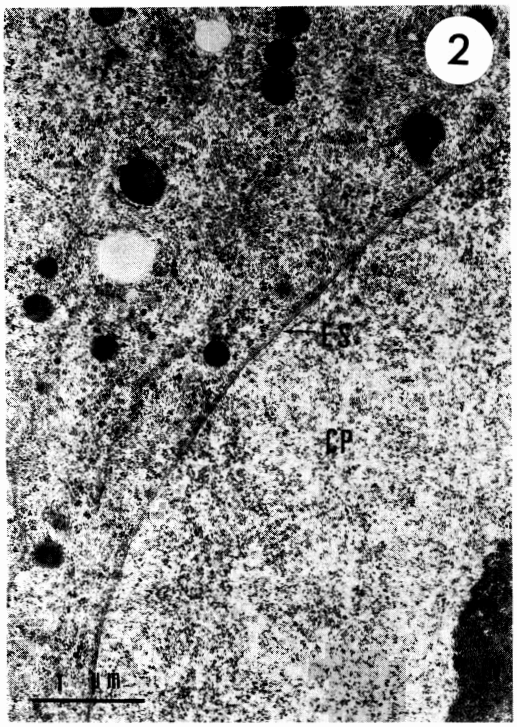
Egg shells were less electron-dense, being as thick as about 40 nm. As no noticeable structures and no homogeneous form were observed on egg shells, it was considered to be still in the course of morphogenesis because of such heterogenous form (Fig. 2).

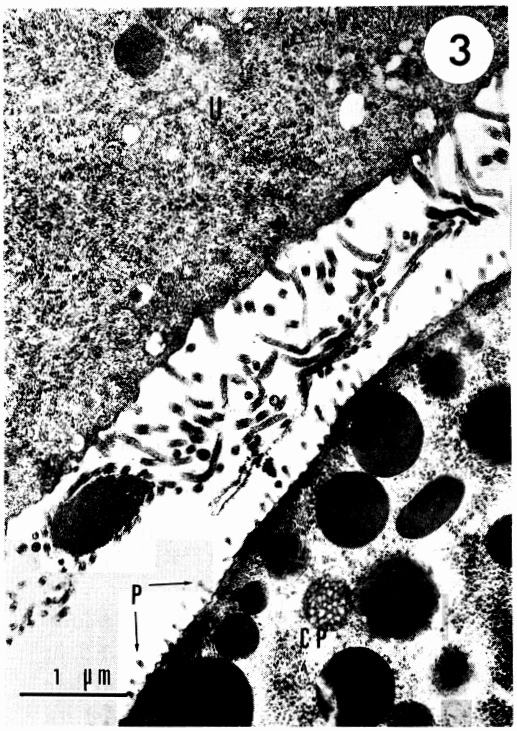
Eggs in Terminal End of Uterus

Fig. 3 shows the eggs adjacent to the uterus. A number of chitinous projections were observed on the surface of the egg shell and those shapes were quite different from the villous-like structures present on the uterine wall. Fig. 4 shows two eggs adjacent to each other. Numerous projections were similarly noted. They were in a club form with the ends somewhat swollen, 150 nm in length, 30 nm at the base and 50 nm at the end in width on an average, and counted 130 per μm^2. Egg shells were noted as being somewhat electron-dense, being about 50 nm in thickness.

Eggs on 1st Day of Cultivation

On eggs in the 1st day of cultivation, 1 to 3 microvilli were seen at the end of almost all projections (Figs. 5 and 6). These microvilli were 150 nm in mean length, 10 nm or less in width and straight and sharply pointed just like


2
ES
CP
1 μm


3
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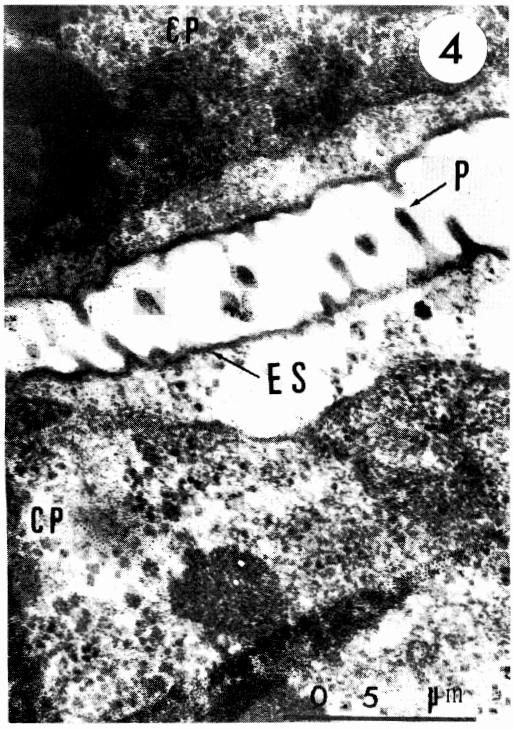
CP
4
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ES
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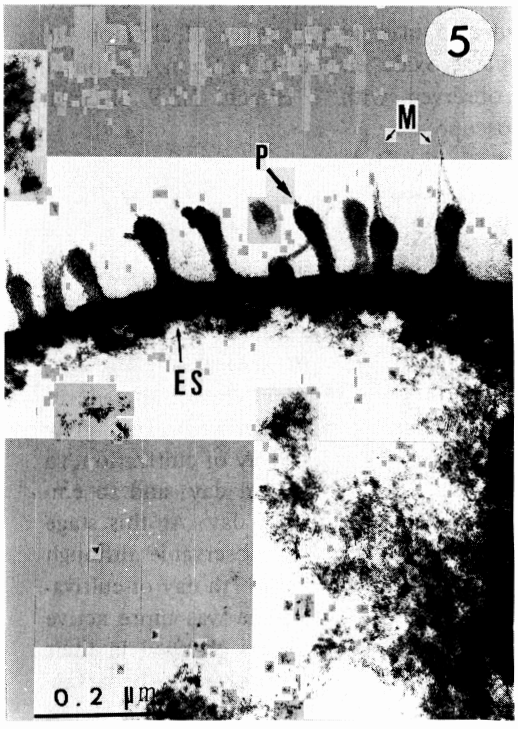
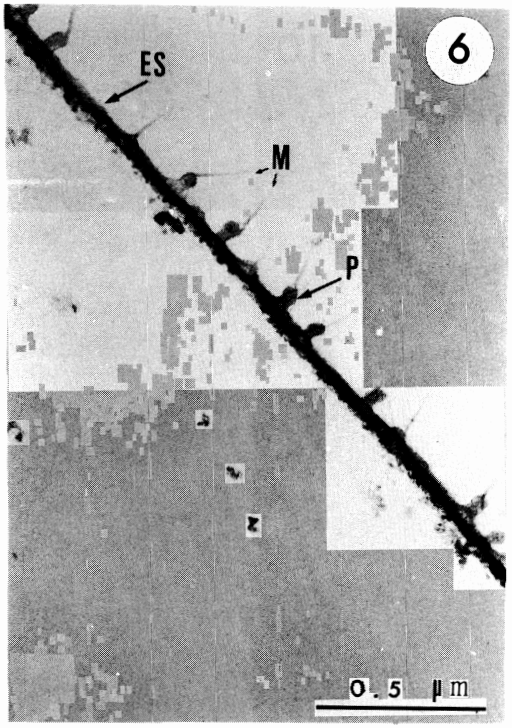
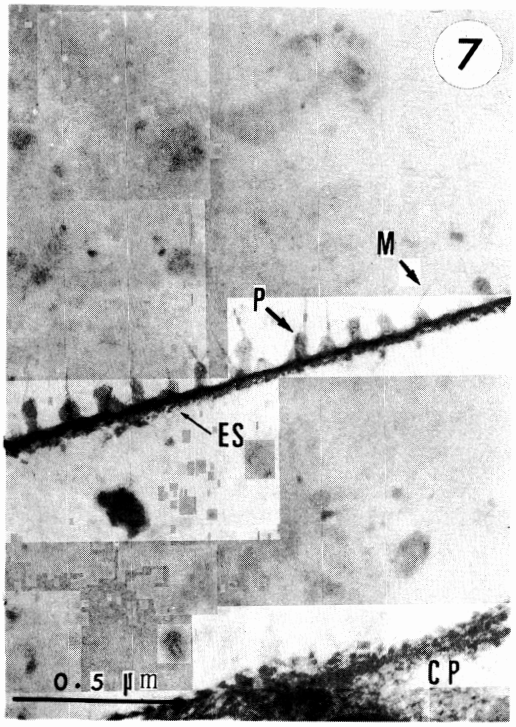
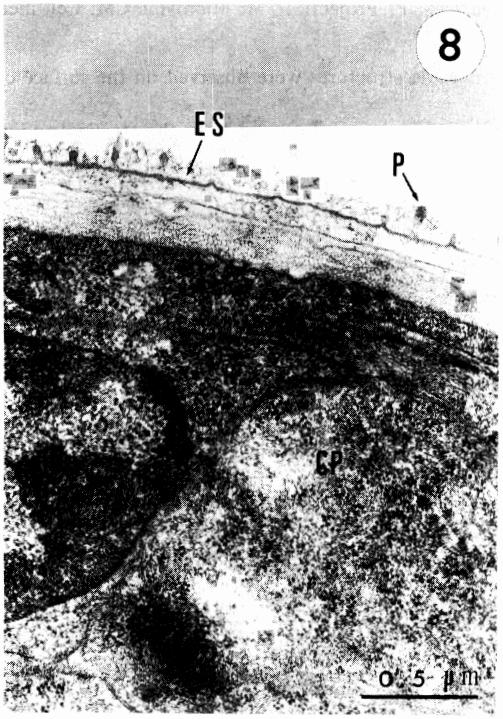
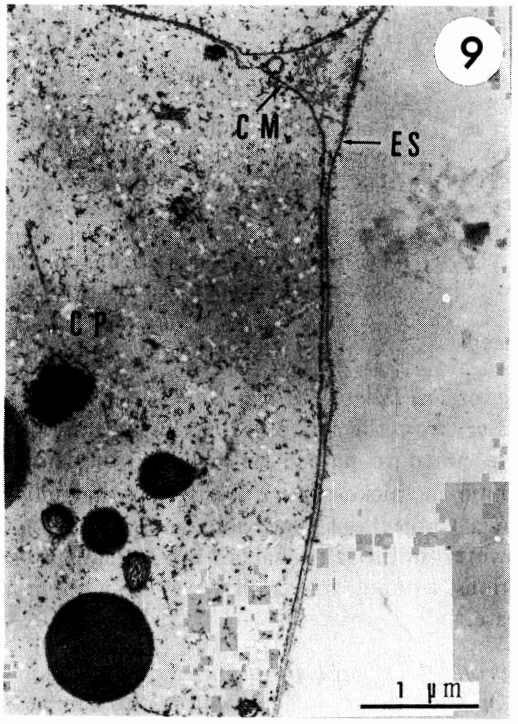
0.5 μm

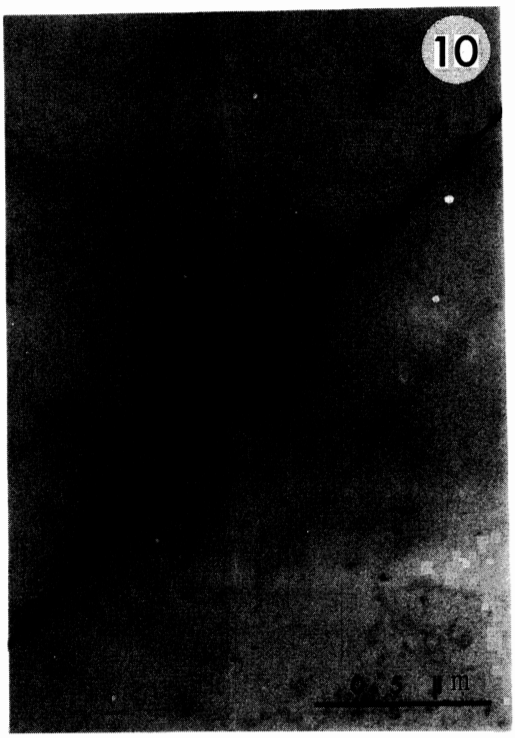

5
M
P
ES
0.2 μm


6
ES
M
P
0.5 μm


7
M
P
ES
0.5 μm
CP


8
ES
P
CP
0.5 μm


9
CM
ES
CP
1 μm



Abbreviations: ES: Egg shell, CP: Cytoplasm, N: Nucleus, P: Projection, M: Microvilli, CM: Cell membrane.

Fig. 2 Egg in the middle part of the uterus. No noticeable structures were observed on the surface of egg shell.

Figs. 3, 4 Chitinous projections of the surface of egg shell. Eggs were recovered from the terminal end of the uterus.

Figs. 5, 6 Eggs from the 1st day of cultivation. Projections and microvilli were seen on the egg shell.

Fig. 7 Egg from the 3rd day of cultivation. Projections and microvilli were seen being fully developed.

Figs. 8, 9 Eggs from the 5th day of cultivation. Projections without microvilli were increased.

Fig. 10 Egg from the 7th day of cultivation. Egg shell reduced its thickness without observation of any microvilli. Projections reduced in their length and numbers.

a needle.

Eggs on 3rd Day of Cultivation

Eggs from the 1st to 3rd day of cultivations were noted to have clear and electron-dense (50 nm in thickness) egg shells with the fully grown projections and microvilli (Fig. 7). However, in the latter half of the 3rd day, projections without microvilli were liable to increase.

Eggs on 5th Day of Cultivation

On the 5th day of cultivation, the number of projections without microvilli further increased (Fig. 8). Eggs without projections were occasionally noted (Fig. 9).

Eggs on 7th Day of Cultivation

Every egg contains a larva. Egg shells are much thinner (10 nm) without showing any microvilli at all. The projections became shorter and much fewer (Fig. 10). Meanwhile, those projections and microvilli observed throughout this series of observations were always homogeneous at any stage of growth, and hardly considered to have any special structure.

Discussion

The observations have been done on the morphology of helminth eggs using the electron microscope, and interesting structures (Hockley, 1968; Inatomi *et al.*, 1970) and difference among species (Ishii, 1972) have been pointed out. Schistosomes have a large lateral spine on the surface of egg shells. The role of this lateral spine in the final host is known to keep eggs in a blood vessel and help them to migrate into tissue (Hockley, 1968; Zaman, 1983). The authors conducted this study with an idea that a structure with similar functions could also be observed in *A. cantonensis* egg shells with the same parasitic behavior as schistosomes which are also intravascular parasites.

As a result two structures which are completely different morphologically were observed (in this study the terms, "projection" and "microvilli", were used based on the morphological characteristics noted individually). The structures changed their shapes in accordance with the growth of the eggs. The eggs in the terminal end of the uterus just before oviposition showed projections first on the surface of egg shells. Within 24 hr after starting cultivation, microvilli were noted at the end of those projections, and such a situation lasted for about 2 days. After then these structures lost their characteristics gradually and on the 7th day of cultivation there remained only a trace.

A similar structure has also been noted in other schistosomes (Hockley, 1968; Inatomi *et al.*, 1970; Sakamoto and Ishii, 1976). According to Hockley (1968) numerous "small spines" are present on egg shells of *S. mansoni* and *S. haematobium*, and their length and width are reported to be 280 nm and 10 – 30 nm in *S. mansoni.* and 220 nm and 50 nm in *S. haematobium*, respectively. Furthermore, Inatomi *et al.* (1970) and Sakamoto and Ishii (1976) reported a similar structure in *S. japonicum* named "microvilli-like projection". Although each report mentions only a single structure, it might be possible to find a new structure by further observations of the eggs at different stages of development. Referring to the functions of the structures observed in schistosomes, Hockley (1968) has stated that they 1) serve to enlarge the surface area of the egg, 2) trap the egg secretions on the surface of the egg shell, and/or 3) have a mechanical function like the large spine. Inatomi *et al.* (1970) have considered it as important behavior relating to the metabolism of the egg. However, the authors are of the opinion that it may not be related to the metabolism of the egg, but rather to the function of blocking and fixing eggs in pulmonary capillaries for the following reasons: (1) the structures observed in *A. cantonensis* were not observed in eggs of other parasites except the intravascular parasites such as schistosomes, (2) the structures started to degenerate and disappear on the 3rd day of cultivation when metabolism of the eggs is still active, and (3) the egg shells consisted of a homogeneous structure, showing no specific organ nor tissue.

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