The Korean Journal of Parasitology
Vol. 34, No. 4, 215-224, December 1996

Localization of cytoskeletal proteins in *Cryptosporidium parvum* using double immunogold labeling

Jae-Ran YU[1]* and Soon-Hyung LEE[2]

Department of Parasitology[1], College of Medicine, Kon-Kuk University, Choongju 380-701,
Department of Parasitology and Institute of Endemic Deseases[2],
Seoul National University College of Medicine, Seoul 110-799, Korea

Abstract: Actin and some actin binding proteins such as tropomyosin, α-actinin and troponin T were localized by simultaneous double immunogold labeling in several developmental stages of *Cryptosporidium parvum*. All of the observed developmental stages have many particles of tropomyosin and actin around pellicle and cytoplasm. Tropomyosin was labeled much more than the actin when these two proteins were labeled simultaneously. And α-actinin was labeled mostly in the pellicle, but troponin T labeling was very rarely observed. From this study, it was suggested that tropomyosin seemed to be one of the major proteins of *C. parvum*, so it must be playing important roles in *C. parvum*.

Key words: Actin, tropomyosin, α-actinin, troponin T, *Cryptosporidium parvum*

INTRODUCTION

Cryptosporidium is one of the major causative agents inducing opportunistic infections in immunocompromised hosts. Severe watery diarrhea is the main symptom and sign which most patients complain. Nevertheless, the effective chemotherapeutic agents have not been developed yet.

In Korea, about 20% of outpatients who visited the Severance Hospital, one of private hospitals in Seoul, were proved to discharge *C. parvum* oocysts in their stools (Cho and Im, 1993). The first human case confirmed by electronmicroscopy was reported in 1995 (Kang *et al.*, 1995).

There are no available data about motility mechanism of coccidia including *C. parvum*. It seems likely that *C. parvum* has no special organ for motility as in flagellates or cilliates. To observe whether *C. parvum* has actomyosin system as in skeletal muscle, distribution of actin and myosin was observed by immunogold labeling in the previous study (Yu and Chai, 1995). In that study, well organized actomyosin system like in muscle could not be seen. Actin and myosin were located mainly along the pellicle in trophozoite stage and labeled actin amount was increased in actively dividing stage such as meront. In the present study, tropomyosin, α-actinin, and troponin T were labeled by immunogold to observe their distribution in parasite cytoplasm and to speculate interrelationships as cytskeletal proteins in *Cryptosporidium*.

• Received Oct. 25 1996, accepted Nov. 14 1996.

*Corresponding author

MATERIALS AND METHODS

1. Activation of *Cryptosporidium parvum* in mice

Cryptosporidium parvum was prepared through immunosuppression of ICR mice weekly with Depomedrol®(10 mg/kg, Korea Upjohn Ltd.). From 1 week after immuno-

suppression, modified acid fast staining was done with mice stools to confirm the oocysts expulsion. The ileum of *C. parvum* induced mice was resected for tissue preparation.

2. Preparation of *C. parvum* tissue antigen

The mouse ileum was fixed in 2% paraformaldehyde and 0.4% glutaraldehyde (pH 7.4) at 4°C for 2 hrs and washed with 0.1 M PBS. Tissues were dehydrated with alcohol, embedded with LR gold resin (Electron Microscopy Sciences) and polymerized at -20°C for 72 hrs under UV light. Ultrathin (90 nm) sectioned specimens were put onto nickel grids.

3. Immunogold labeling for electron microscopy

1) Actin and tropomyosin labeling: Sectioned tissues were incubated with primary antibodies mixed IgG fraction of mouse monoclonal antibody raised against chiken gizzard actin (Chemicon) and rabbit polyclonal antibody raised against chicken gizzard tropomyosin (Sigma) at 4°C overnight. The specimens were washed thoroughly in PBS-BT (1% bovine serum albumin and 0.01% Tween 20 in PBS) and then followed by secondary reaction with 10 nm gold-conjugated goat anti mouse IgG (BioCell) and 5 nm gold-conjugated goat anti rabbit IgG (BioCell) simultaneously for 2 hrs at room temperature. They were washed again in PBS-Tween and incubated at room temperature for 15 min with 2.5% glutaraldehyde to stabilize gold particles. For silver enhancement, Amersham enhancement kit were used (Amersham Ltd.). After uranyl acetate and lead citrate staining, electronmicroscopic observations were done with Jeol 1200 EXII. To rule out the steric hindrance which may be induced by using smaller particles in tropomyosin labeling than in actin at this study, we did another one with exchanged gold sizes each other.

2) Tropomyosin and α-actinin labeling: Mixed rabbit polyclonal antibody raised against chicken gizzard tropomyosin (Sigma) and mouse monoclonal antibody raised against chicken gizzard α-actinin (Sigma) were used as primary antibodies. For secondary antibodies, 5 nm gold-conjugated goat anti rabbit IgG (BioCell) and 15nm gold-conjugated goat anti mouse IgM (BioCell) were used.

3) Tropomyosin and troponin-T labeling: Mixed rabbit polyclonal antibody raised against chicken gizzard tropomyosin (Sigma) and mouse monoclonal antibody raised against rabbit skeletal muscle troponin T (Sigma). For secondary labeling, 5 nm gold-conjugated goat anti rabbit IgG (BioCell) and 10nm gold-conjugated goat anti mouse IgG (BioCell) were used.

RESULTS

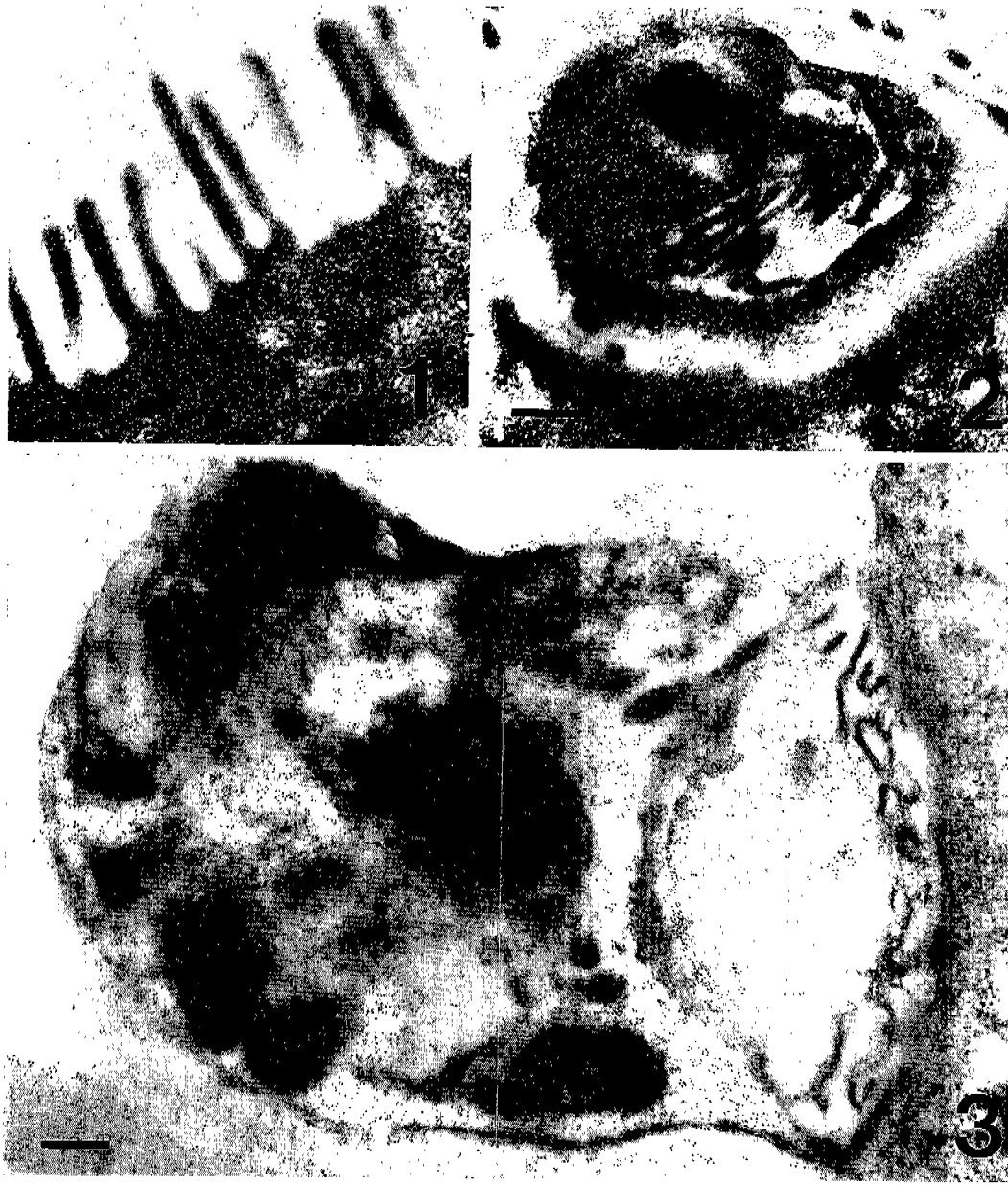
1. Actin and tropomyosin labeling

All of the observed developmental stages of *Cryptosporidium parvum* in specimens showed a lot of labeled tropomyosin on their cytoplasm as well as on the pellicles (Figs. 1-8). The meront stage has more amount of tropomyosin than in trophozoites (Figs. 3 & 4). But the nucleus, electron-dense bodies and the area around feeder organelle and parasite-host junction (electron-dense band) were showed much less amount of gold labeling. On the other hand, the number of gold particles labeled at the parasite actin were also much less than that of tropomyosin. Host actin were obserbed at terminal web region under the parasite-host junction (Fig. 3). The amount of labeled tropomyosin was as much as enough to identify *C. parvum* very easily with low magnifying power of electron microscope (Fig. 8). Through the second study using exchanged gold size between tropomyosin and actin each other, we obtained the same result as the first study (Figs. 5-7).

By the way, in case of accidentally found *Giardia muris* had very few gold particles against tropomyosin and actin than in *C. parvum*. Only the vacuoles around the flagellum were labeled by gold particles (Figs. 9 & 10).

2. Tropomyosin and α-actinin labeling

Tropomyosin and α-actinin located along the pellicle of developing meront, but α-actinin was labeled very rarely in cytoplasm (Fig. 11). Type I meront had not much α-actinin, however it had a lot of tropomyosin in its

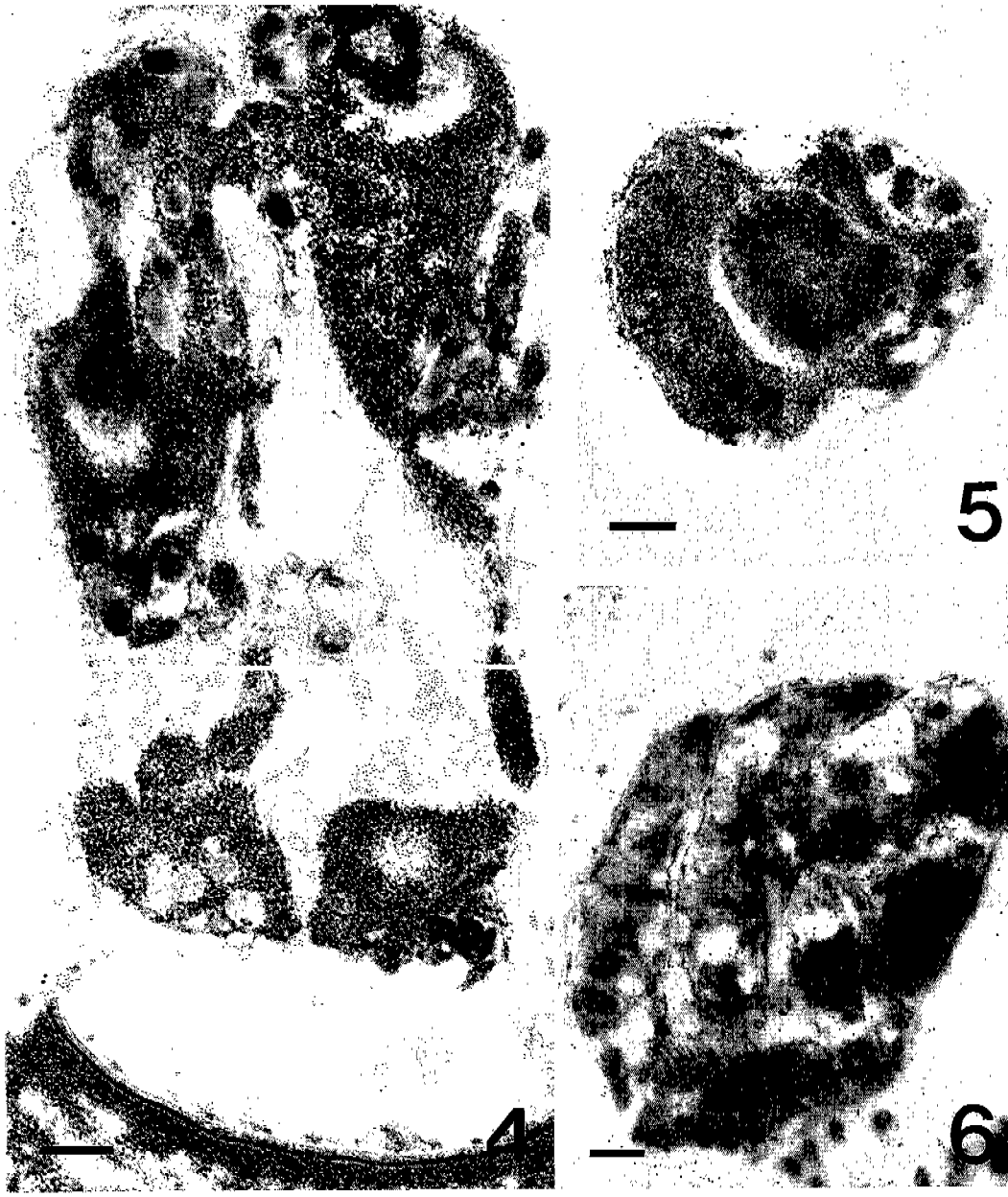


Figs. 1-3. Double immunogold labeling at actin and tropomyosin (TM) in *C. parvum*. The mouse small inestinal brush border was labeled with gold particles (Fig. 1). Trophozoite stage (Fig. 2). Type I meront: both pellices and cytoplasm showed tropomyosin labeling. Host actin particle were labeled under the electro-dense junction. 5 nm, TM; 10 nm, actin (Fig. 3). Bar, 200 nm.

cytoplasm (Fig. 12). Also in Type II meront, α-actinin and tropomyosin were located along its pellicle, but in the cytoplasm of merozoites the amount of tropomyosin was not as much as in type I meront (Fig. 13).

3. Tropomyosin and troponin-T labeling

Both of trophozoite and type I meront were not labeled by troponin T (Figs. 14 & 15). It was suggested that *C. parvum* had very few amount of troponin T in its cytoplasm.



Figs. 4-6. Double immunogold labeling at actin and TM in *C. parvum*. Developing meronts. 5 nm, TM; 10 nm, actin (Fig. 4). Trophozoites and type I meronts showed many gold particles labeled at TM. 5 nm, actin; 10 nm, tropomyosin (Fig. 5-6). Bar, 200 nm.

DISCUSSION

Cryptosporidium actin is most smilar to the γ-isoform of vertebrate cytoplasmic actin (Kim *et al.*, 1992). One of actin accessory proteins is tropomyosin, and the other major accessory protein in vertebrate skeletal muscle is troponin. These proteins control the skeletal muscle contraction under the presence of Ca^{++}. Skeletal muscle type tropomyosin stabilizes and stiffens the actin filaments, inhibits binding of filamin to actin filaments, so increases the binding of myosin II to the

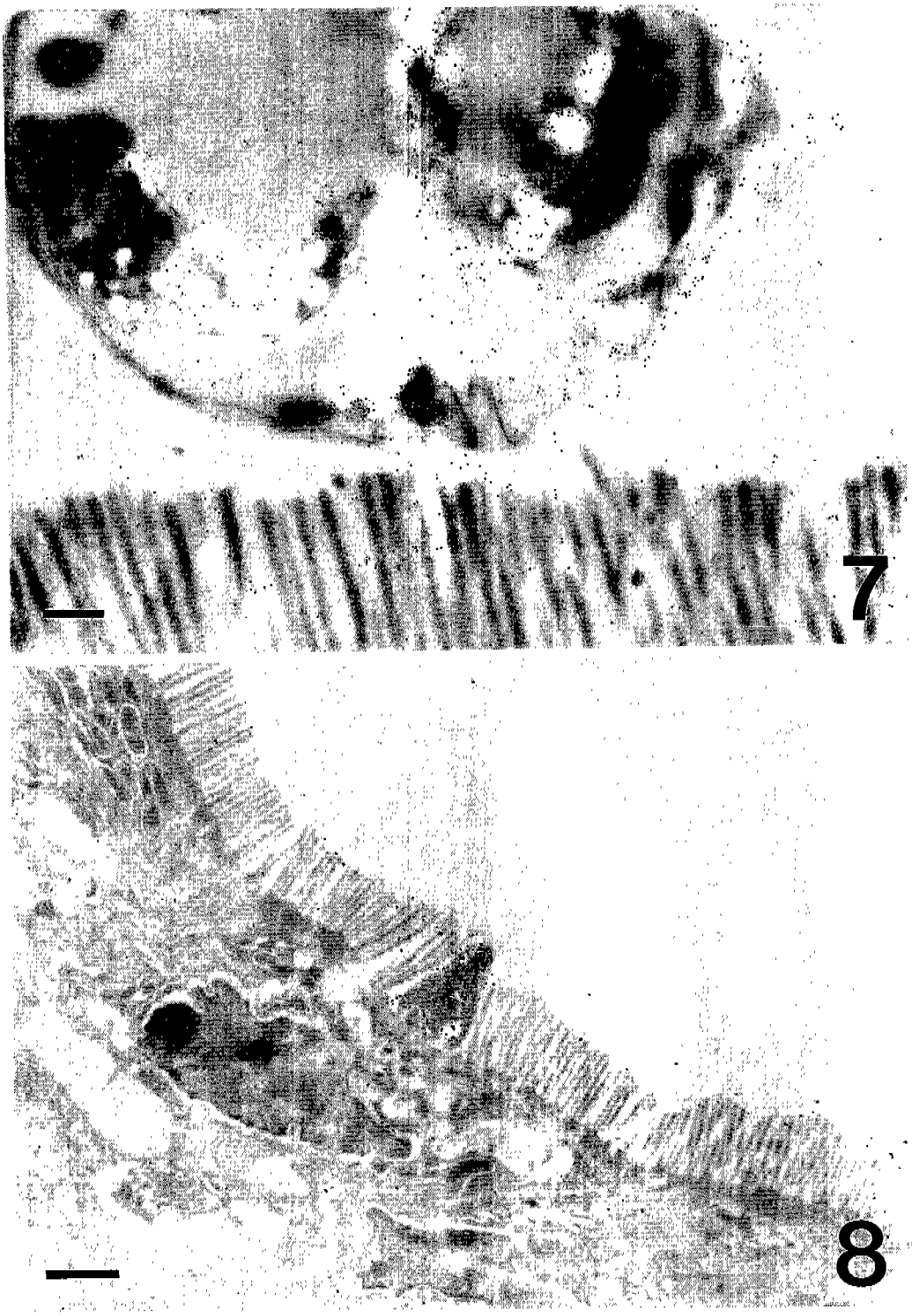
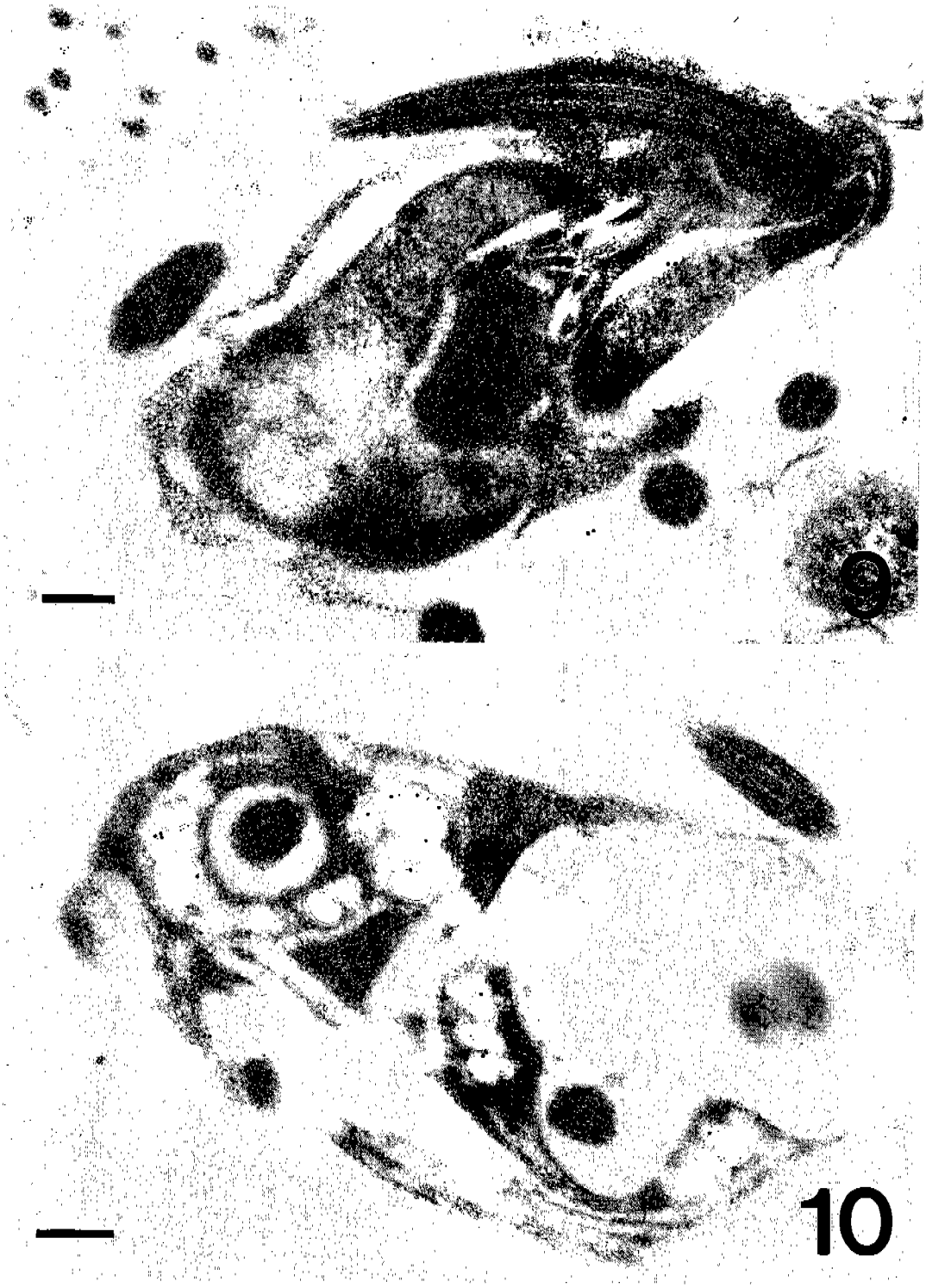


Fig. 7. Double immunogold labeling at actin and TM in oocyst of *C. parvum*. 5 nm, actin; 10 nm, TM. Bar, 200 nm. **Fig. 8.** Immunogold labeling of only TM with silver enhancement in *C. parvum*. x 5000. Bar, 1 μm.



Figs. 9-10. *G. muris* immunogold labeled at TM and actin simultaneously. Labeled gold particles were much less than in *C. parvum*. Vesicles around the flagellum were mostly labeled by gold (Fig. 10). 5 nm, TM; 10 nm, actin. Bar, 200 nm.

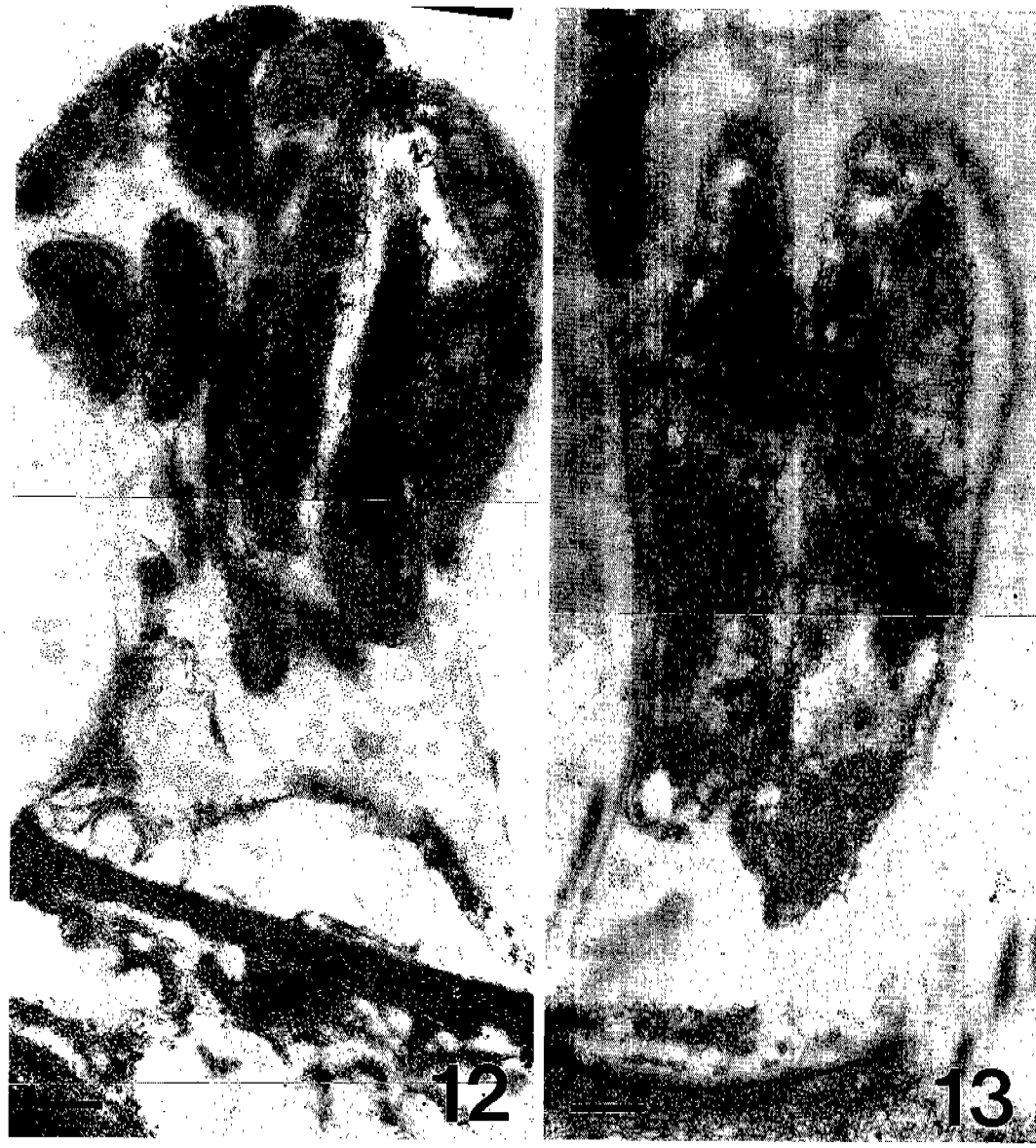
Fig. 11. Double immunogold labeling at TM and α-actinin in developing meronts of *C. parvum*. α-actinin was located mainly around the parasite pellicle and the amount was much less than TM. 5 nm, TM; 15 nm, α-actinin. Bar, 200 nm.

filament. Non muscle type tropomyosin involved in actin-filament-linked regulatory system for the mechanisms of cell division, cell motility, phagocytosis, pinocytosis, exocytosis, cytoplasmic streaming, but precise function is unknown (Alberts *et al.*, 1994). While these systems are thought to be largely controlled by the calcium-dependent phosphorylation of the myosin light chains and do not appear to contain troponin (Lau *et al.*, 1985).

α-actinin is also one of the actin bundling proteins associated in making loosely packed actin bundles allow myosin II. It is concentrated in stress fibers, where it is thought to be partly responsible for the relatively loose cross-linking of actin filaments in these contractile bundles; it also helps to form the anchorage for the ends of stress fibers where they terminate on the plasma membrane at focal contacts (Alberts *et al.*, 1994).

In this study, the results showed that *C. parvum* has abundant amount of tropomyosin, even more than the amount of actin. The amount of tropomyosin in *C. parvum* is as much as enough to detect it very easily with low magnifying power (x 5000) of electron microscope. So, tropomyosin must be associated with lots of biological phenomena besides maintaining the parasite architecture. And probably the number of gold particles labeled at tropomyosin is so many that the real site of actin can not be localized as like as in the previous study (Yu and Chai, 1995). The location of tropomyosin and actin might be very similar. Host actin was localized under the electron-dense band area between host and parasite, but host tropomyosin could not be seen from there.

Whereas in flagellates, which was accidentally found during study, tropomyosin does not seem to be as important as in *C. parvum* through the number of labeled gold particles. It was supposed that tropomyosin

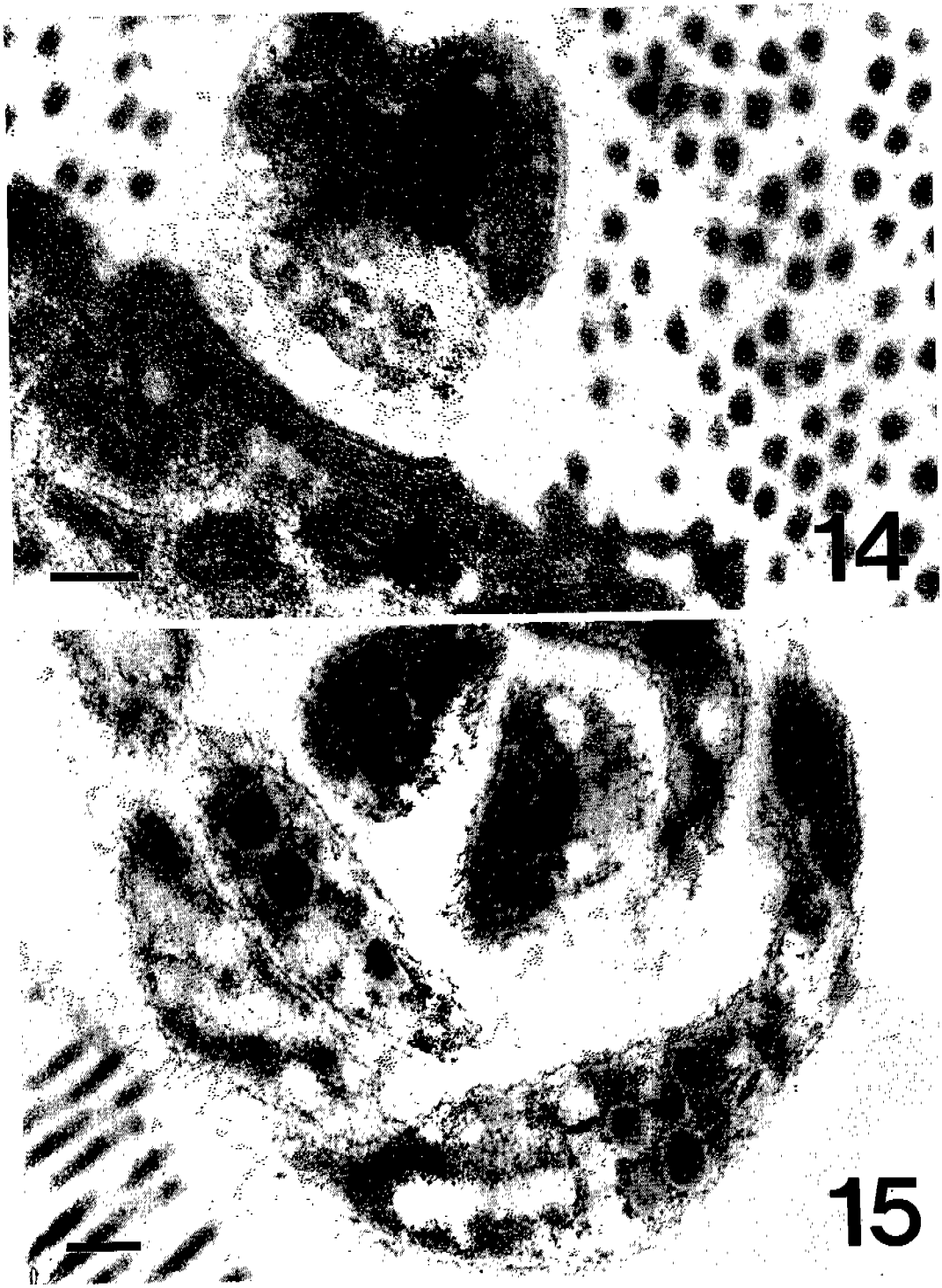


Figs. 12-13. Double immunogold labeling at TM and α-actinin in type I (Fig. 12) and type II meronts (Fig. 13). 5 nm, TM; 15 nm, α-actinin. Bar, 200 nm.

and actin of *G. muris* related with the transportation of vesicles in cytosplasm because of their labeled location around vesicles.

In this study, it was very hard to see labeled troponin T. There are two possibilities to explain the result. One reason is that the amount of troponin T is extremely little or the other one is tropomyosin labeling masks the antigen site of troponin T.

Schistosoma mansoni tropomyosin is known to have significant homology to host molecules, but it is species specific and does not seem to cross-react with sera from patients with *S. haematobium* and *S. mansoni* or with other parasitic diseases. As well as, the tropomyosin gene product in *S. mansoni* is strongly associated with the tegument and also with the muscle of adult worms. Adult schistosome parasites turn over their



Figs. 14-15. Double immunogold labeling at TM and troponin-T in trophozoites (Fig. 14) and type I meronts (Fig. 15). Labeled troponin-T can hardly be seen. 5 nm, TM; 10 nm, troponin-T. Bar, 200 nm.

membrane every 3-6 hr and this may be the source of antigen for the host immune system (McLaren, 1980). Also, the species specificity of *S. mansoni* tropomyosin has a potential as an immunodiagnostic reagent (Xu *et al.*, 1991). In case of *Cryptosporidium*, the parasite pellicle is released after merozoites or sporozoites escape from their mother, then the actin, tropomyosin and α-actinin aggregated in the pellicle can be the immunogen to the host as in case of *Schistosome*. So it seems likely that these cytoskeletal proteins can be used as the target of immunodiagnostic or immunotherapeutic materials.

REFERENCES

Alberts B, Bray D, Lewis J, Raff M, Roberts K, Watson JD (1994) Molecular biology of the cell, 3rd ed. p787-861, Garland Publishing Inc, New York & London.

Cho MH, Kim AK, Im KI (1993) Detection of *Cryptosporidium* oocysts from out-patients of the Severance Hospital, Korea. *Korean J Parasitol* **31**(3): 193-199.

Kang YK, Lee HK, Kim SW, Chi JG (1995) Cryptosporidiosis in a leukemia child with severe diarrhea. *Seoul J Med* **36**(1): 29-34.

Kim K, Gooze L, Petersen C, Gut J, Nelson G (1992) Isolation, sequence and molecular karyotype analysis of the actin gene of *Cryptosporidium parvum*. *Mol Biochem Parasitol* **50**: 105-114.

Lau SYM, Sanders C, Smillie LB (1985) Amino acid sequence of chicken gizzard γ-tropomyosin. *J Biol Chem* **260**(12): 7257-7263.

McLaren DJ (1980) *Schistosoma mansoni*: The parasite surface in relation to host immunity. Research Studies Press, New York.

Xu H, Rekosh DM, Andrew W, Higashi G, Nicholson L, Loverde PT (1991) *Schistosoma mansoni* tropomyosin: production and purification of the recombinant protein and studies on its immunodiagnostic potential. *Am J Trop Med Hyg* **45**(1): 121-131.

Yu JR, Chai JY (1995) Localization of actin and myosin in *Cryptosporidium parvum* using immunogold staining. *Korean J Parasitol* **33**(3): 155-164.

=초록=

이중면역황금표지법을 이용한 작은와포자충의 세포골격 단백질 분포 관찰

유재란[1], 이순형[2]

건국대학교 의과대학 기생충학교실[1], 서울대학교 의과대학 기생충학교실 및 풍토병연구소[2]

작은와포자충의 세포골격 단백질 분포를 알아보기 위하여 actin, tropomyosin, α-actinin 및 troponin-T의 분포를 이중면역황금표지법(double immunogold labeling method)으로 관찰하였다. 관찰된 모든 발육 단계의 충체에서 매우 많은 양의 tropomyosin이 세포질 및 세포막에 분포하고 있음이 밝혀졌으며 actin보다도 더 많은 양이 관찰되었다. α-actinin의 경우는 주로 세포막에 소량이 분포하였으며 Troponin-T는 어느 발육 단계에서도 관찰되지 않았다. 이 연구의 결과 tropomyosin의 분포 정도도 미루어 볼 때 이 단백질이 작은와포자충에서 매우 중요한 역할을 수행할 것으로 추측되며, 주로 세포막에 다량으로 분포하는 actin, tropomyosin 및 α-actinin은 면역진단 및 면역치료의 대상으로 이용할 가능성이 있을 것으로 생각된다.

(기생충학잡지 34(4): 215-224, 1996년 12월)