The Korean Journal of Parasitology
Vol. 33, No. 3, 155-164, September 1995

Localization of actin and myosin in *Cryptosporidium parvum* using immunogold staining

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Abstract: The location of actin and myosin of the several stages of *Cryptosporidium parvum* was observed. The tissue antigen of *C. parvum* was prepared through immunosuppression of ICR mice with Depomedrol®. The thin sectioned specimens, which were incubated with the IgG fraction of the rabbit polyclonal antibodies raised against chicken back muscle actin and bovine uterus myosin, were treated with 10 nm gold-conjugated goat anti-rabbit IgG. Electrodense particles were located mainly on the pellicles of all observed developmental stages of the parasites. The number of actin gold particles in the cytoplasm increased when the parasite was dividing actively as in case of meronts. Especially in macrogametocytes, a lot of actin and myosin particles were synthesized and storaged as amilopectin-like bodies. There were many actin gold particles along the microspikes of cytoplasmic membranes in various developmental stages. The actin and myosin observed in this study may play important roles to control the shape of the parasites and movements of cytoplasmic membranes as cytoskeletal proteins.

Key words: *Cryptosporidium parvum*, actin, myosin, cytoskeletal proteins

INTRODUCTION

Cryptosporidiosis had been recognized as an animal parasitic disease since Tyzzer discovered *Cryptosporidium muris* from mouse stomach, and it was not considered as a human parasitic disease before the first human cases were published in 1976 (Neim, 1976; Meisel, 1976). However, recently it became one of the most important opportunistic protozoal diseases and often life-threatening in immunocompromised human hosts. In Korea, several species of *Cryptosporidium* were found from various hosts (Chai *et al.*, 1990; Lee *et al.*, 1991a & 1991b) and the first human case was confirmed by pathology specimen of the appendix which was resected from a leukemic patient (Kang *et al.*, 1995).

Because *C. parvum* is a monogenous parasite, its all developmental forms could be seen in the small intestinal epithelium. So merogony and gametogony could be often observed together at near sites. During these complex life cycle stages of *C. parvum*, so many essential processes for survival should occur such as host cell invasion of zoites, multiple division of merozoites and sporozoites, and fertilization of gametocytes. The cytoskeletal proteins were known to be involved in these essential processes in other highly evoluted animal cells (Alberts *et al.*, 1994), but not in *C. parvum*. We evaluated in this study the locations of actin, which is one of the most

• Received Aug. 4 1995, accepted after revision Aug. 26 1995.

• This study was supported by a Research Grant for Basic Medical Science, Ministry of Education, Republic of Korea Government (1994).

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important cytoskeletal proteins, and myosin, motor protein of actin, to get the suggestions for the rols of cytoskeletal proteins in the survival of *C. parvum.*

MATERIALS AND METHODS

1. Activation of *Cryptosporidium parvum* in mice

ICR mice (3 weeks) were immunosuppressed by subcutaneous injection of Depomedrol® (Korea Upjohn Ltd.), 5 mg/kg per week for three weeks. Modified acid fast staining on the mice stools was done every three days to confirm oocyst evacuation. The ileums of mice, confirmed by oocyst expulsion, were resected at 3 weeks after immunosuppression.

2. Preparation of *C. parvum* tissue antigen

The mice ileum were fixed with the mixture of 2% paraformaldehyde and 0.4% glutaraldehyde (pH 7.4), washed with 0.1 M PBS, and dehydrated through alcohol series. The dehydrated tissues were embedded in LR gold resin (Electron Microscopy Sciences) and polymerized at -20°C for 72 hrs under UV light. The tissue was sectioned at 90 nm thickness by ultramicrotome and mounted onto nickel grids.

3. Immunogold staining for electron microscopy

The sectioned specimens were washed in PBS-Milk-Tween (3% skim milk and 0.01% Tween 20 in PBS) for 10 min. They were incubated for 3 hrs at room temperature with the IgG fractions of rabbit anti-actin (chicken back muscle actin, BioCell) and anti-myosin (bovine uterus myosin, BioCell) polyclonal antibodies. The immunoreacted specimens were washed thoroughly in PBS-BT (1% bovine serum albumin and 0.01% Tween 20 in PBS) and then reincubated at 4°C overnight with a 10 nm colloidal gold conjugated goat anti-rabbit IgG (Biogenex). They were washed again in PBS-Tween and incubated at room temperature for 15 min with 2.5% glutaraldehyde to stabilize gold particles. They were washed in PBS and stained with uranyl acetate and lead citrate and examined under a transmission electron microscope (Jeol 1200 EXII).

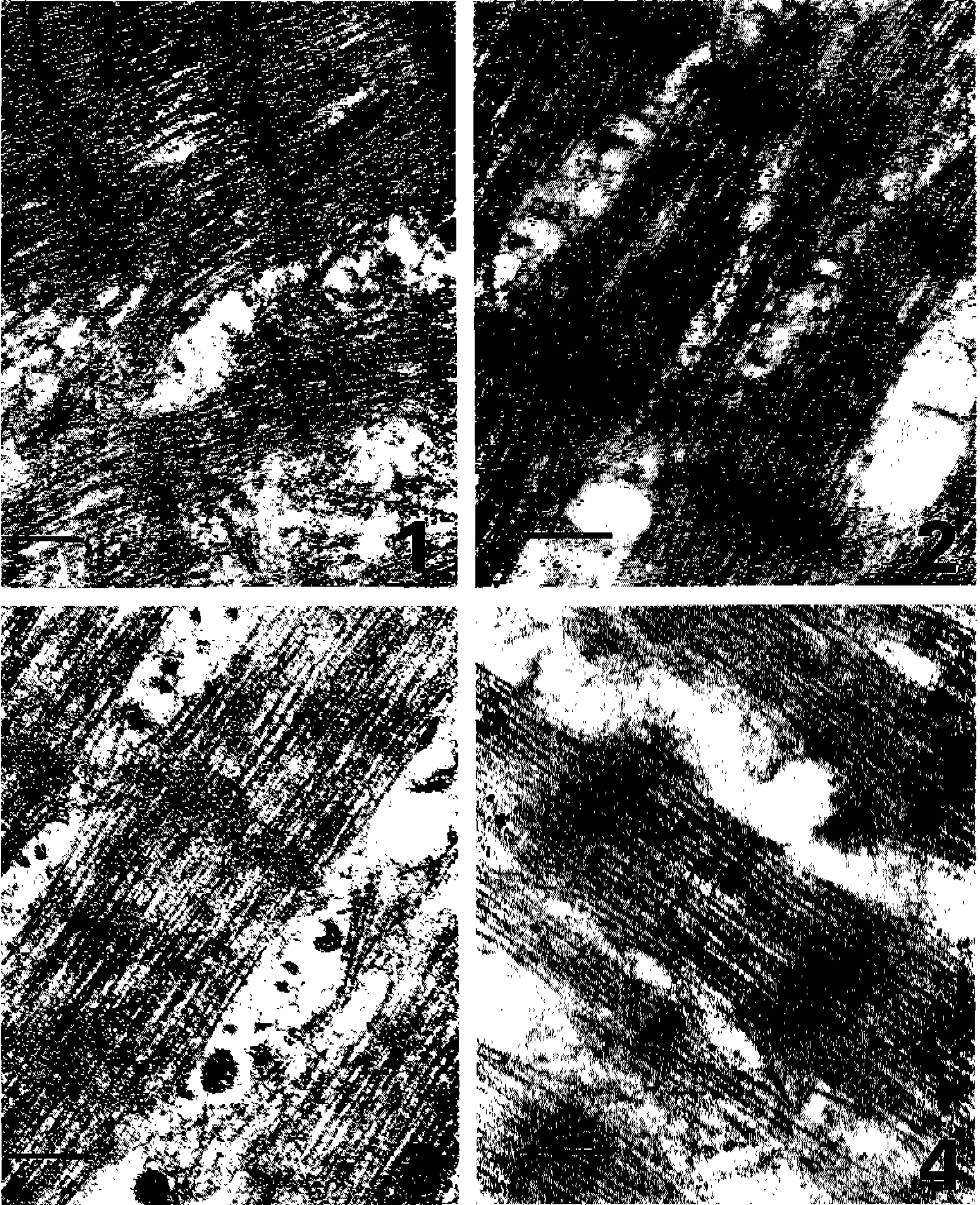
RESULTS

1. The localization of actin

The primary antibody to chicken back muscle actin recognized well the actin of the rat abdominal muscles (Fig. 1 & 3). In the trophozoites of *C. parvum* stained with the immunogold method, gold particles were seen along pellicles and cytoplasm near feeder organelles, but not on the nucleus and cytoplasm near the luminal side (Fig. 5). In the uninucleated meront, gold particles were distributed all over the cytoplasm and membranes (microvillous, parasitophorous and parasite membranes) except the nucleus and dense bodies, and their number increased more than in trophozoites (Fig. 6). In the type II meront containing 4 merozoites, gold particles were located mainly on the pellicle of the meront and membranes of merozoites rather than the cytoplasm. The residual bodies in the remnant space between merozoites in the meront had many gold particles also (Fig. 7 & 10). In the macrogametocytes, amylopectin-like bodies showed many gold particles, while wall forming bodies did not show any particles. Also the cytoplasm around the parasite-host junction and membranes were stained well (Fig. 8). In the developing oocysts, the gold particles were located around the membranes also and in the center of the cytoplasm which seemed to be in the process of mitosis (Fig. 9). Fully matured oocyst including only one remained sporozoite showed gold particles on the oocyst wall and membranes of sporozoites (Fig. 11). Empty parasitophorous vacuoles, from which all merozoites must have escaped, were seen sometimes (Fig. 12 & 13). Some of them showed microspikes stained with gold particles and long projections like filopodia as if they were microvilli of the small intestinal epithelium (Fig. 12).

2. The localization of myosin

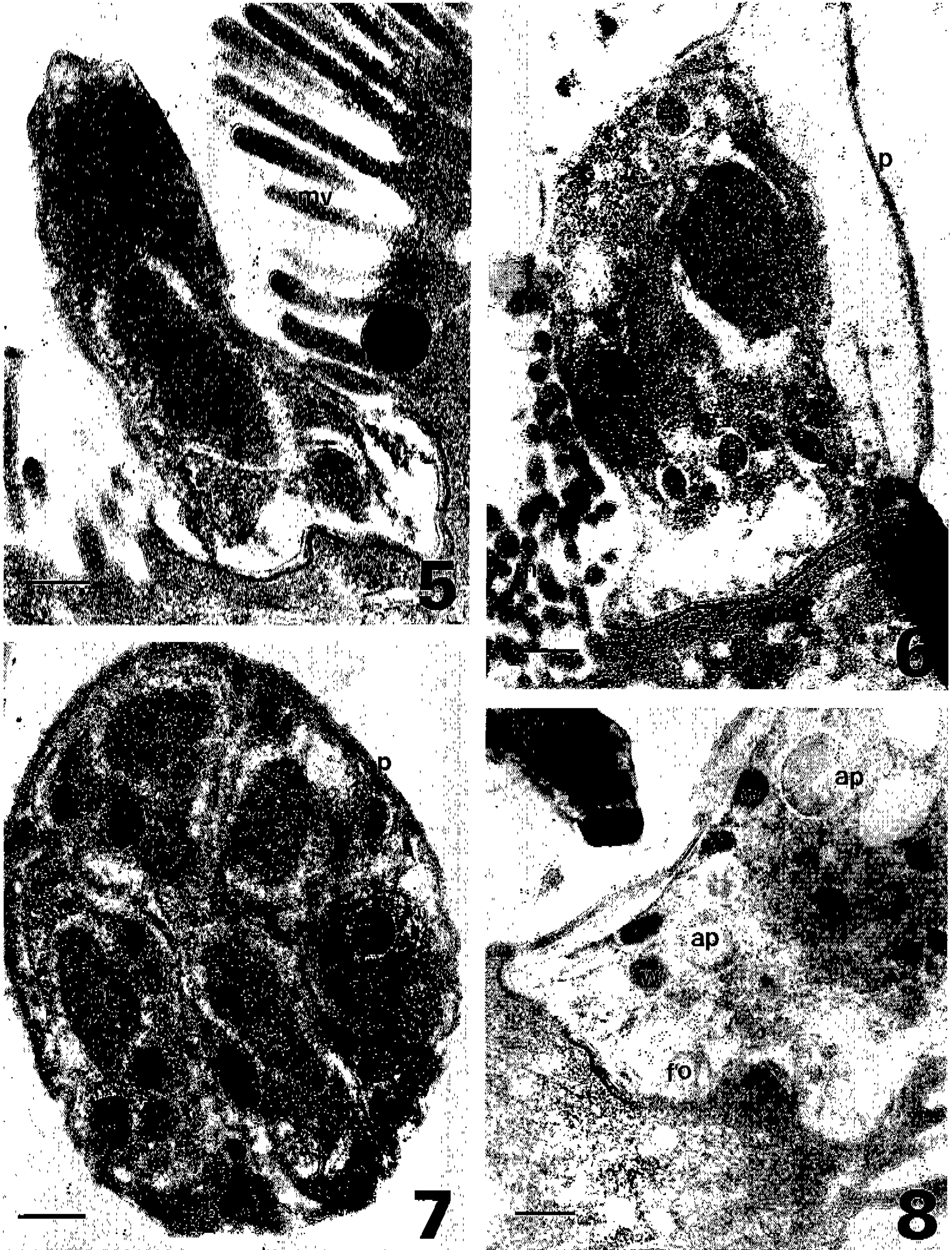
The primary antibody to bovine uterus myosin recognized well the myosin of the rat abdominal muscles, so the gold particles localized specifically to the muscle bundles



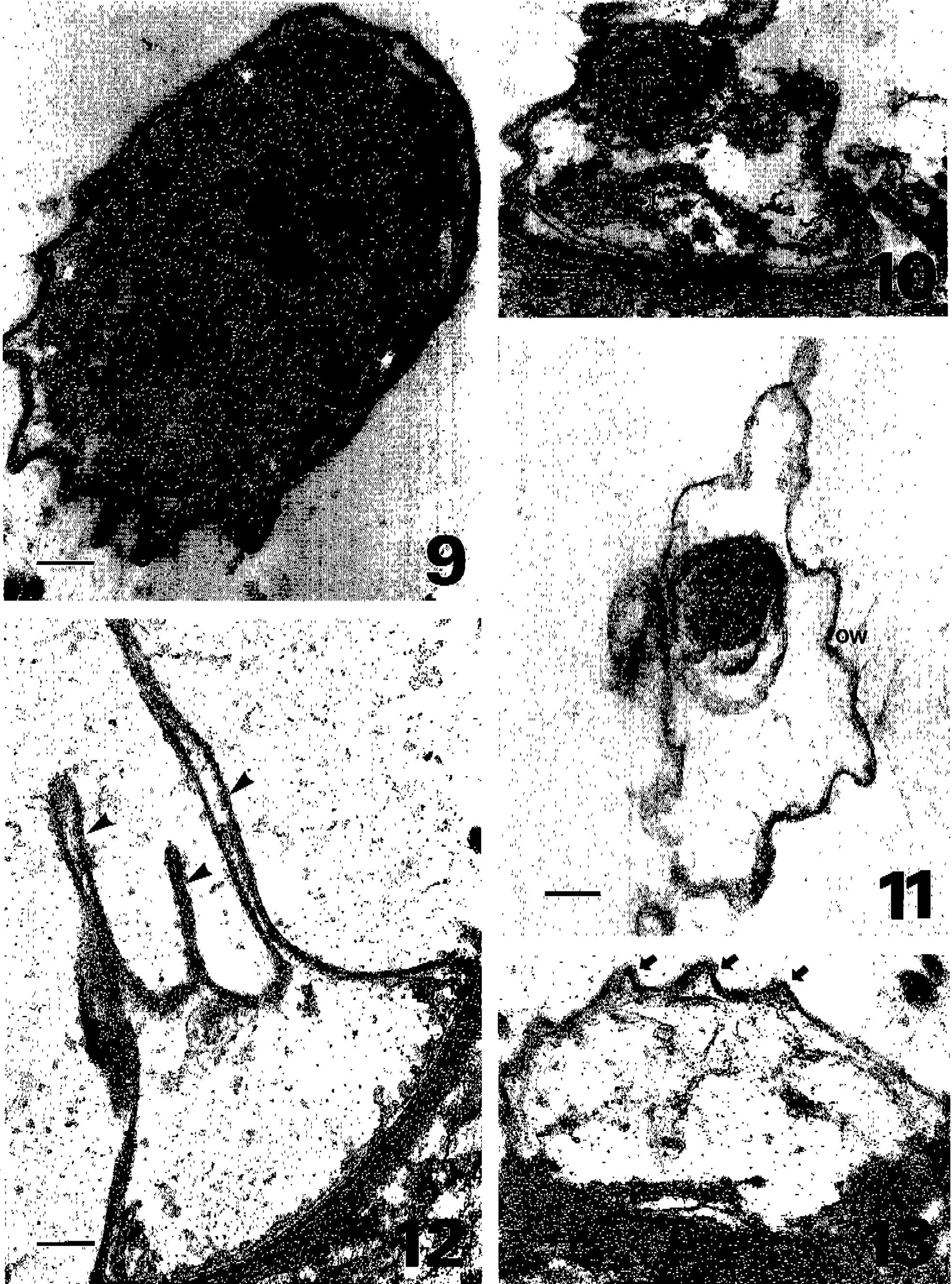
Figs. 1-4. Positive control of immunogold staining with primary antibodies against actin (**Fig. 1**) and myosin (**Fig. 2**) in the rat abdominal muscle. Negative control of immunogold staining missing primary antibodies against actin (**Fig. 3**) and myosin (**Fig. 4**). Bar; 200 nm.

(Fig. 2 & 4). In the trophozoites of immunogold stained specimens, gold particles were attached on the pellicle, but they were not on the cytoplasm and nucleus (Fig. 14). In the uninucleated meronts, the distribution pattern of gold particles was the same as trophozoites (Fig. 15). By the way, the number of gold particles far increased in the macrogametocytes (Fig. 16). The myosin was accumulated at the same location, amylopectin-like bodies, as in the case of actin. In the type II meront, a few gold particles were localized on the pellicle of the meront and membranes of the merozoites.

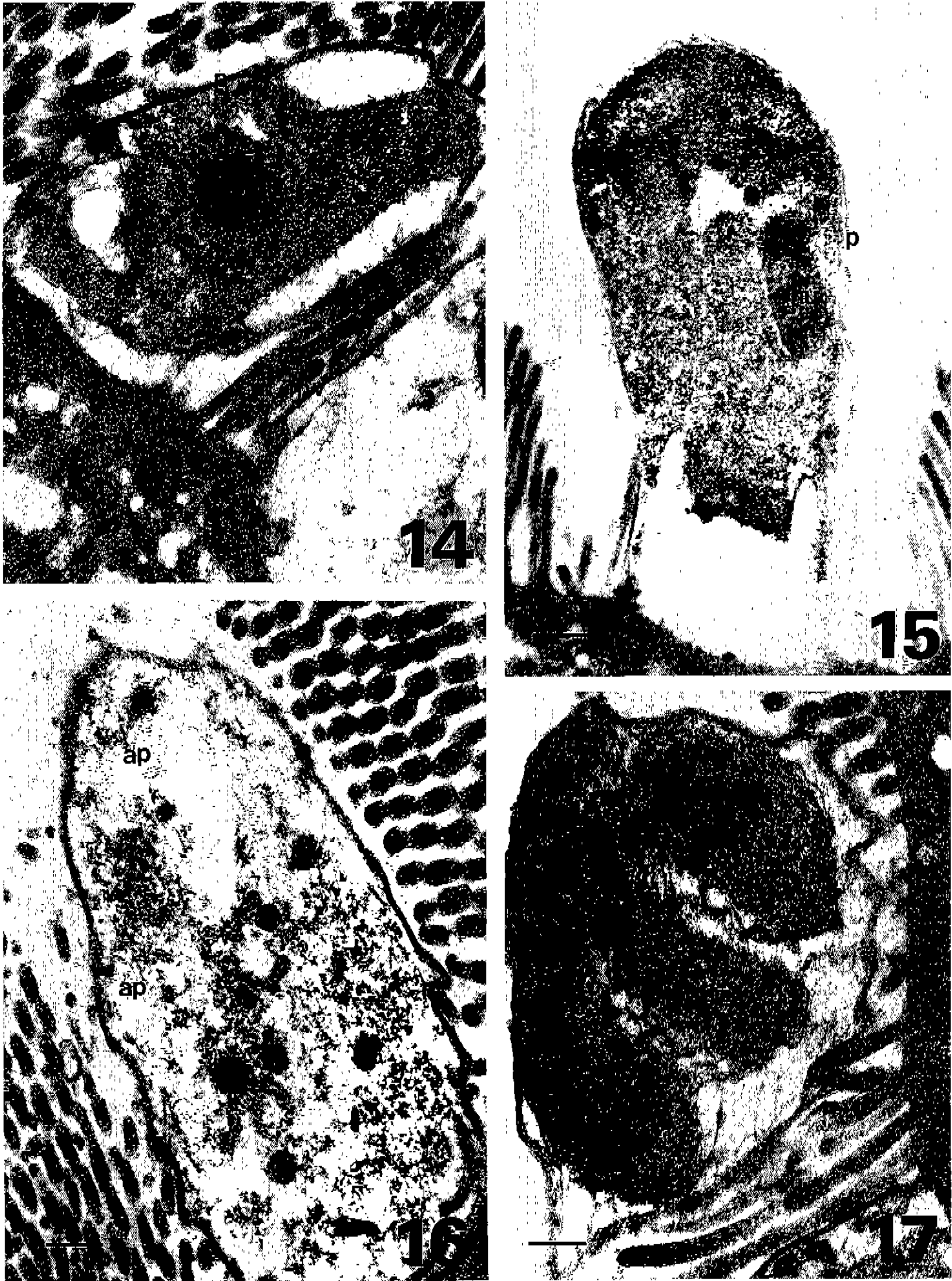
In dividing oocysts, gold particles were located at the same location (Fig. 17 & 18).



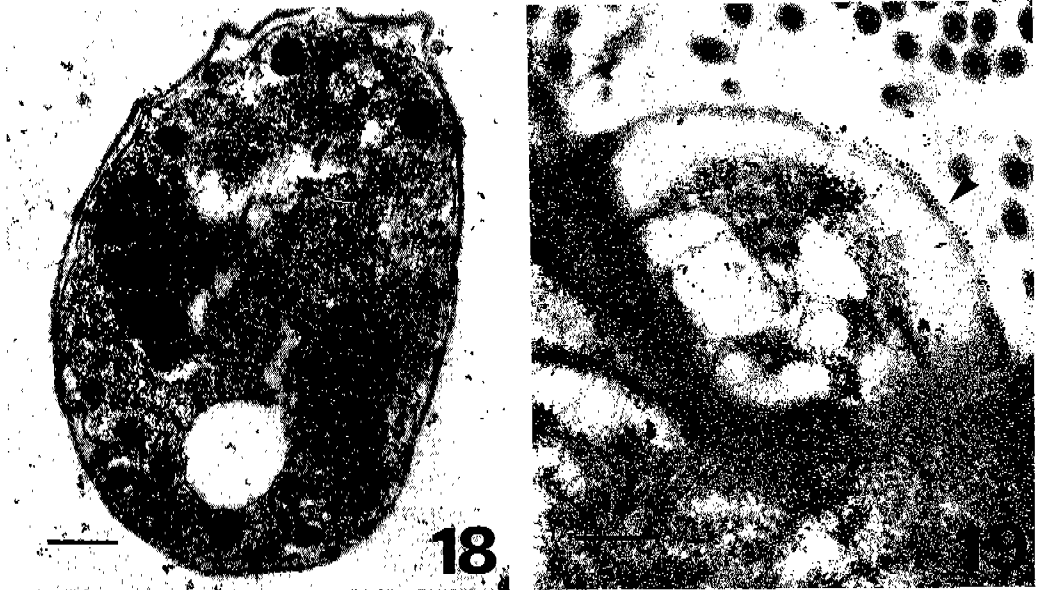
Figs. 5-8. The localities of actin shown by immunogold staining. In the trophozoite, gold particles are seen on the pellicle and cytoplasm around the feeder organelle (**Fig. 5**). In the uninucleated meront, gold particles are dispersed all over the cytoplasm and pellicle except the nucleus and dense bodies (**Fig. 6**). In the type II meront, the pellicle and membranes between the merozoites have gold particles (**Fig. 7**). In the macrogametocyte, amylopectin-like bodies and cytoplasm around the feeder organelle show many gold particles, whereas wall forming bodies do not (**Fig. 8**). ap; amyolpectin-like body, d; dense body, fo; feeder organelle, mv; microvilli, nu; nucleus, p; pellicle, r; rhoptry, rb; residual body, w; wall forming body. Bar; 200 nm.



Figs. 9-13. The localities of actin shown by immunogold staining. In the developing oocyst, gold particles are located along the oocyst wall and cytoplasmic membrane and the actin detected at the center of the cytoplasm (asterisk) is thought to be involved in mitosis of the cell (**Fig. 9**). In the mature meront including one unescaped merozoite, the pellicle, merozoites membrane and cytoplasmic residuum show many gold particles (**Fig. 10**). Mature oocysts containing one sporozoite show the same pattern as mature meronts (**Fig. 11**). Some vacant parasitophorous vacuoles reveal long projectons like filopodia (arrow head) and microspikes (arrow) and they have many actin gold particles (**Fig. 12, Fig. 13**). cr; cytoplasmic residuum, mz; merozoite, ow; oocyst wall, p; pellicle, sp; sporozoite. Bar; 200 nm.



Figs. 14-17. The localities of myosin shown by immunogold staining. In the trophozoite (**Fig. 14**) and uninucleated meront (**Fig. 15**), gold particles are detected along the pellicles, but they are very few in the nucleus and cytoplasm. In the macrogametocyte, amylopectin-like bodies are stained well with gold particles (**Fig. 16**). In the type II meront, gold particles are distributed along the pellicle and merozoites membranes also (**Fig. 17**). ap; amylopectin-like body, mv; microvilli, nu; nucleus, p; pellicle, r; rhoptry, w; wall forming body. Bar; 200 nm.



Figs. 18-19. The localities of myosin shown by immunogold staining. In the developing oocysts a small number of gold particles were detected along the oocyst wall (**Fig. 18**). In an unidentified developmental stage, gold particles showed well the localization of myosin along the pellicle (arrow head; **Fig. 19**). Bar; 200 nm.

Some specimens showed well the localization of myosin on the pellicle (Fig. 19).

DISCUSSION

The cytoplasm of eukaryotic cells is spatially organized by a network of protein filaments known as the cytoskeleton. This network contains three principal types of filaments: microtubules, actin filaments, and intermediate filaments. Actin filaments form the cortex just beneath the plasma membrane with a variety of actin-binding proteins. This actin-rich layer controls the shape and surface movements of most animal cells (Alberts *et al.*, 1994). Actin filaments are also responsible for the cell crawling movement, muscle contraction and intracellular movements such as the transport of organelles from one place to another in the cytoplasm and the segregation of chromosomes at mitosis (Alberts *et al.*, 1994).

Cryptosporidium parvum has various developmental stages within the intestinal epithelium of the host. Many biological, chemical and mechanical activities occur during the life cycle such as zoites motility, host cell invasion, protrusion from the host cells, division of the meront and fertilization process between gametocytes. So it seems likely that cytoskeletal proteins including actin filaments play an important part in the life cycle of *C. parvum*. So probably actin has the key for survival of *C. parvum*.

In this study, actin and myosin were detected from all observed developmental stages of *C. parvum*, but the actin gold particles were dominant. Actin and myosin were located mainly along the pellicles of the parasites, so according to their location they seemed to be involved in making the shapes of parasites and in membrane surface movement such as protruding of microspikes. As the matter of fact, each stage of *C. parvum* was thought to move continuously within the epithelial cells through formation of microspikes, and some of them were as long as enough to be misunderstood as microvilli. In the meront stages, the remarkably increased actin gold particles gave the suggestions that when the cell is dividing, not only polymerization of actin monomer but also synthesis of actin was promoted. Especially in the meront including merozoites, actin gold

particles located principally along the cytoplasmic membranes of merozoites. It looks likely that actin filaments have relation with making the cytoplasmic membrane of the merozoites by means of forming constriction rings between dividing merozoites as in other animal cells (Alberts *et al.*, 1994). Besides the cytoplasmic membrane, the residual bodies in the remnant space of meront appeared to have many actin gold particles, but the roles of them were not easy to speculate.

The actin around the feeder organelle in the trophozoites or macrogametocytes might form the gel-like network and control the transport of materials obtained from the host cells, through the lamellipodia-like movement of the crawling cell, but it must be proved by other methods (Stossel, 1994). It was a quite unexpected finding that there were many gold particles representing actin and myosin in amylopectin-like bodies of macrogametocytes. The exact meaning of the existence of actin and myosin in this organelle was very hard to be explained. Probably the amylopectin-like bodies serve as protein storage units in which various kinds of proteins as well as actin and myosin were stored.

The primary anti-myosin antibody used in this study could detect both myosin I on the microvilli of the small intestinal epithelium and myosin II on the rat abdominal muscle. The myosin gold particles were attached along the cytoplasmic membranes in all developmental stages of *C. parvum*, whereas intracytoplasmic organelles, nucleus, and cytoplasm showed very few gold particles. Because non-muscle type myosin is called type I and rather primitive than the muscle type, the myosin detected in *C. parvum* was thought to be the type I, and it may be involved in transporting the small vesicles and supporting the shape of the parasites as an attaching motor protein at the actin cortex as it usually does in animal cells (Alberts *et al.*, 1994).

In this study, we could hardly get any suggestion about participation of acto-myosin contractile system to the motility of zoites in *C. parvum* for the exact median cut sections could hardly be found. We have the plan to do further study on the location of actin and myosin of zoites. Cytochalasin B or D, depolymerizing agent of actin filaments, inhibited the motility of *Toxoplasma gondii* and *Eimeria* sp., that means actin takes part in their motility (Russel and Sinden, 1981). Especially actin was located at the anterior part such as the conoid, preconoidal ring and polar ring, and anterior cytoplasmic membrane in *T. gondii* (Schwartzman and Pfefferkorn, 1983).

In this study, the actin filaments in various stages of *C. parvum* were assumed to play many important roles. First, they may control the surface movement as well as the shapes of parasites by locating along the cytoplasmic membrane with myosin. Second, they may be related to cell division such as merozoites formation in meronts by increasing polymerization of the filaments as well as synthesis. Finally, it may have an effect on exchange and transportation of some materials between the parasite and host cell by forming gel-like structure around the feeder organelle. Conclusively, actin molecule must be an essential material on the survival of *C. parvum*, so if we could differentiate the actin molecule of *C. parvum* from that of humans, we can use it beneficially to control *C. parvum*.

ACKNOWLEDGEMENTS

The authors thank Dr. S. Huh in Department of Parasitology, College of Medicine, Hallym University for his interest and advice during this study. We also thank Mr. B.W. Chang (Department of Electron microscopy, College of Medicine, Kon-Kuk University) and Mrs. J.H. Lee (Department of Elecron microscopy, College of Medicine, Chungbuk National University) for their kind and delicate technical supports.

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=초록=

작은와포자충에서 면역황금염색법을 이용한 actin과 myosin의 위치 관찰

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*Cryptosporidium parvum*의 발달 단계별 actin과 myosin의 분포 위치를 면역황금염색법을 이용하여 관찰하였다. Depomedrol®을 ICR마우스에 피하주사하여 면역억제시킨 후 *C. parvum*이 발현된 마우스 회장을 잘라 LR gold로 포매하여 초박절편을 떴다. 일차항체로는 chicken back muscle actin과 bovine uterus myosin에 대한 rabbit polyclonal antibody를 사용하였고 이차항체로는 10 nm 크기의 황금입자가 결합된 goat anti-rabbit IgG를 반응시켰다. Uranyl acetate와 lead citrate로 염색한 후 투과전자현미경으로 관찰하였다. Trophozoite에서는 세포막에서 주로 actin과 myosin이 관찰되었고 feeder organelle 주위 세포질에는 actin이 분포하였다. Meront와 같이 활발히 분열하고 있는 단계에서는 세포막과 세포질 전체에 actin이 분포되어있었으며 myosin은 세포막에서만 소량 관찰되었다. 핵과 anlage of rhoptries 등은 두 단백질에 모두 염색되지 않았다. Macrogametocyte에서는 amylopectin-like bodies에서 actin과 myosin이 모두 관찰되었으나 wall forming bodies에서는 관찰되지 않았고 feeder organelle 주위 세포질 부분에서는 actin이 관찰되었다. Sporozoite를 포함하는 oocyst와 merozoite를 포함하는 meront에서는 세포막과 세포막사이에서 actin이 다수 관찰되었으며 myosin은 소량 관찰되었다. Merozoites가 빠져나가 속이 비어있는 parasitophorous vacuole 중에는 microspike를 형성한 것들이 종종 관찰되었고 이것이 좀더 길어져 마치 microvilli와 같이 보이는 경우도 있었으며 이러한 구조물에서도 actin이 다수 관찰되었다. 이상의 결과로 미루어 actin과 myosin은 세포막에 주로 분포하면서 *C. parvum*의 형태를 유지시키며 또한 세포막의 움직임을 조절하는 cytoskeletal protein으로서의 역할이 주된 작용일 것으로 생각되었다.

〔기생충학잡지 33(3): 155-164, 1995년 9월〕