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ELECTRON MICROSCOPIC OBSERVATION OF THE INVASION PROCESS OF *CRYPTOSPORIDIUM PARVUM* IN SEVERE COMBINED IMMUNODEFICIENCY MICE

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ABSTRACT: *Cryptosporidium parvum* mainly invades the intestinal epithelium and causes watery diarrhea in humans and calves. However, the invasion process has not yet been clarified. In the present study, the invasion process of *C. parvum* in severe combined immunodeficiency (SCID) mice was examined. Infected mice were necropsied; the ilea were double-fixed routinely and observed by scanning and transmission electron microscopy. In addition, the microvillus membrane was observed by ruthenium red staining. Scanning electron micrographs showed elongation of the microvilli at the periphery of the parasite. The microvilli were shown to be along the surface of the parasite in higher magnification. Transmission electron microscopy confirmed that the invading parasites were located among microvilli. Parasites existed in the parasitophorous vacuole formed by the microvillus membrane. The parasite pellicle attached to the host cell membrane at the bottom of the parasite, and then the pellicle and host cell membrane became unclear. Subsequently, the pellicle became complicated and formed a feeder organelle. In addition, invasion of the parasite was not observed in either a microvillus or the cytoplasm of the host cell. Therefore, *C. parvum* invades among microvilli, is covered with membranes derived from numerous microvilli, and develops within the host cell.

Cryptosporidium parvum invades the microvilli of gastrointestinal epithelial cells and causes watery diarrhea in humans and calves. This infectious disease is usually self-limited in immunocompetent people, while it becomes chronic in immunocompromised individuals, such as those infected with the human immunodeficiency virus (HIV) (O'Donoghue, 1995; Clark, 1999; Chen et al., 2002). Since the first case was reported in 1976, sporadic infections and outbreaks of human cryptosporidiosis have been reported throughout the world (Chen et al., 2002; Shimura, 2002). Therefore, *C. parvum* is important as a zoonotic pathogen and an emerging infectious disease.

The invasion of coccidian zoites into host cells has mainly been studied using species of *Toxoplasma* or *Eimeria* (Roberts et al., 1970; Dubremetz, 1988, 1998); however, the invasion of *C. parvum* is not thoroughly understood. It has been reported that sporozoites or merozoites enter into the microvillus of an epithelial cell (Iseki, 1979) and that the protozoan is enveloped by redundant folds of membrane after they invaginate the microvilli (Marcial and Madara, 1986). Chen et al. (1998) also observed the attachment and invasion of *C. parvum* sporozoites into cultured cells using time-lapse video microscopy and scanning electron microscopy. However, the detailed invasion process of *C. parvum* has not yet been shown morphologically.

In the present study, the invasion process of *C. parvum* into ileum epithelial cells in SCID mice was examined by scanning and transmission electron microscopy. In addition, the membrane surrounding the parasites was observed by ruthenium red staining.

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MATERIALS AND METHODS

Oocysts

C. parvum of cattle used in this study have been maintained by passage in SCID mice in Department of Infectious Diseases, Kyorin University School of Medicine, Tokyo, Japan. Oocysts from feces of SCID mice infected with *C. parvum* were collected and concentrated by the sugar centrifugal flotation method (Fujino et al., 2002), after being stored in 2% potassium dichromate solution at 4 C. They were then used within 1 mo.

Inoculation of mice

SCID mice (5- to 10-wk-old, males) were used in the present study. They were raised in a coccidian-free environment and confirmed to be free of natural coccidial infections by the sugar flotation method prior to the experiments; 1×10^7–10^8 oocysts were inoculated into each SCID mouse after washing with distilled water (DW) and centrifugation at 670 *g* for 7 min.

Scanning electron microscopy

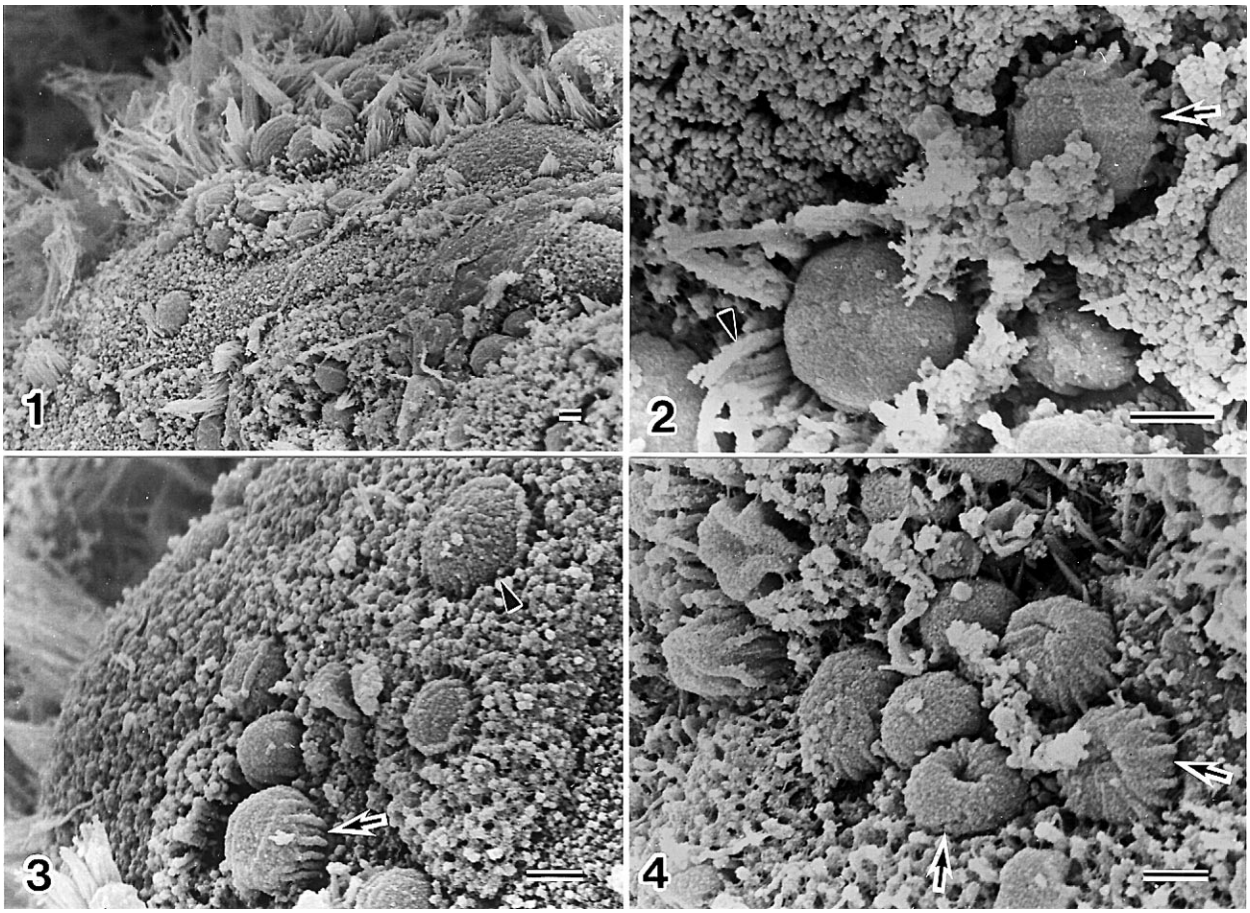
The ilea were removed from inoculated mice at 6, 12, and 18 hr postinoculation. Small pieces of the ilea were fixed with 2.5% glutaraldehyde-2% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4) at 4 C for 4 days and washed in PB. They were postfixed with 1% OsO_4 in PB at 4 C for 2–3 hr and dehydrated in an ethanol series. The specimens were freeze-dried and then gold-coated before the observation with a scanning electron microscope (JSM-5600LV, JEOL Ltd., Tokyo, Japan).

Transmission electron microscopy

The ilea were removed from mice at 18 hr, on 40 and 100 days postinoculation. Small pieces of the ilea were fixed with 2.5% glutaraldehyde in PB at 4 C for 1–4 days and postfixed with 1% OsO_4 in the same buffer at 4 C for 2–3 hr after washing with PB. They were dehydrated in an ethanol series and embedded in Epon resin. Thin sections were stained with uranyl acetate and lead citrate before observation with a transmission electron microscope (JEM-1010, JEOL Ltd., Tokyo, Japan).

Ruthenium red staining

Ruthenium red, which stains the glycocalyx on the surface of microvilli (Takada and Hirano, 1983), was used to observe the relationship between the parasites and these surface structures. Small pieces of the ilea of mice at 40 days postinoculation were fixed with 3.6% glutaraldehyde-ruthenium red (15 mg/DW 10 ml) in 0.2 M cacodylate buffer (CB, pH 7.4) at 4 C for 1 day and washed with 0.1 M CB. They were postfixed with 2% OsO_4-ruthenium red (15 mg/DW 10 ml) in CB at room temperature for 3 hr. The subsequent embedding and observation methods were carried out as described above.



FIGURES 1–4. Scanning electron micrographs showing the surface of the ileum of SCID mice experimentally infected with *C. parvum*. All bars = 1 μm. (**1**) *Cryptosporidium parvum* attached on the apical surface of the epithelial cells. (**2**) The microvilli elongate along the surface (arrow) and at the periphery of (arrowhead) parasites. (**3**) Microvilli which elongated along the surface of the parasite (arrow). The surface of some parasites appears to be bumpy (arrowhead). (**4**) A hole or a hollow is observed at the upper part of the parasites (arrows).

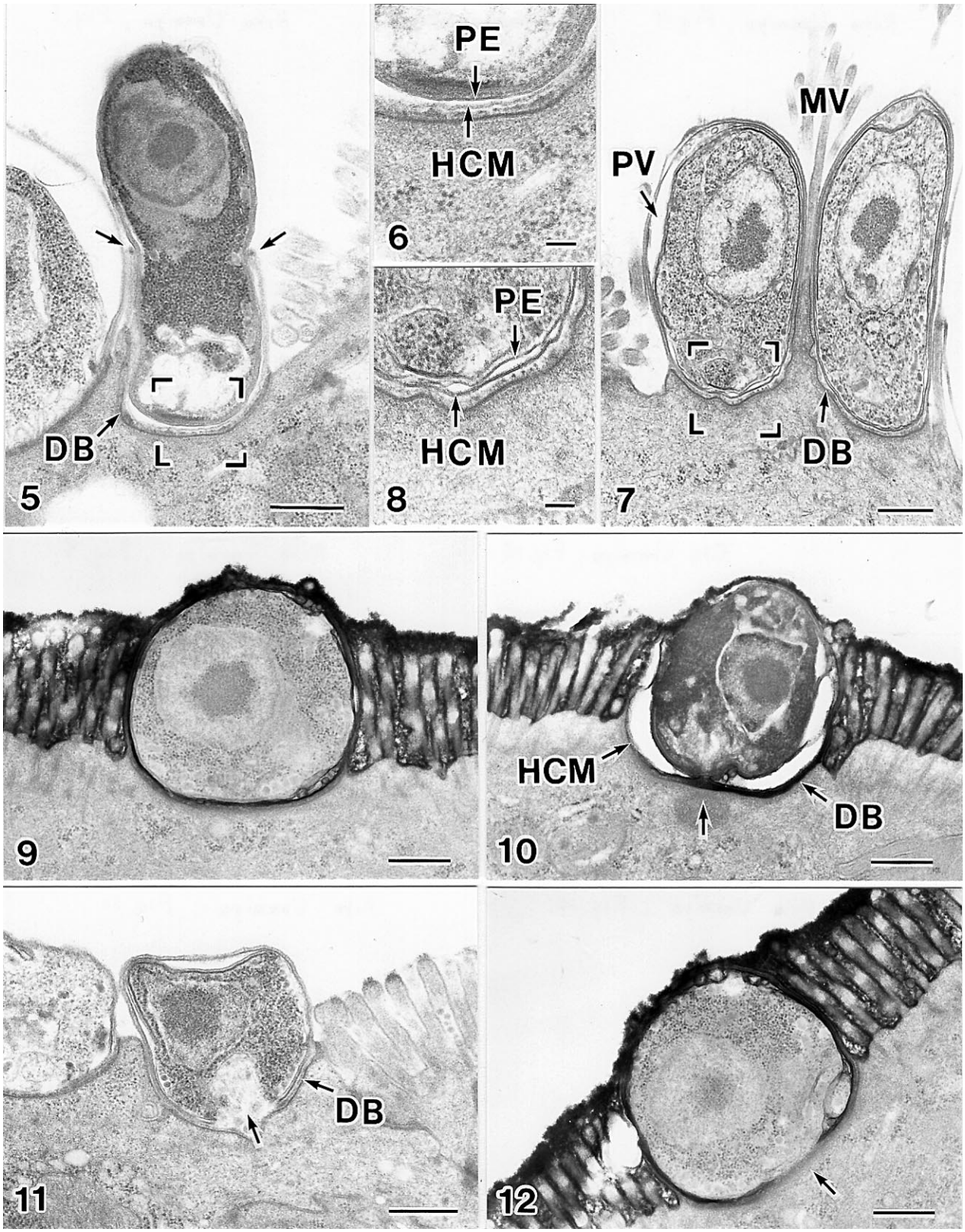
RESULTS

The appearances of parasitism

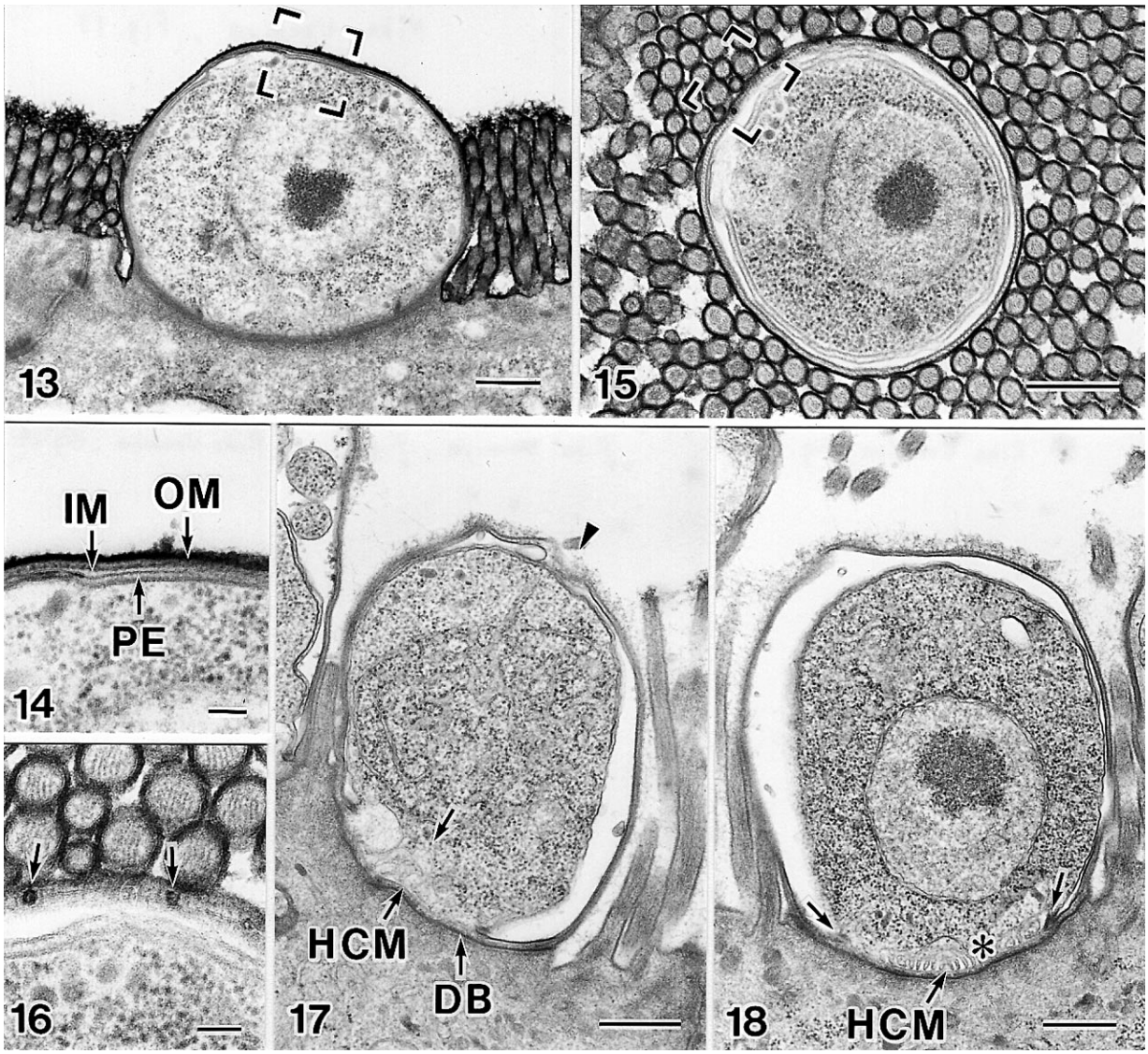
Scanning electron micrographs are shown in Figures 1–4. Most parasites were spherical, and the microvilli of epithelial cells consisting of villi to which parasites attached were elongated just around the parasites (Fig. 1). Elongating microvilli enveloped the parasites (Fig. 2). The surface of some zoites appeared to be uneven (Fig. 3). A hole or a hollow was also observed at the upper part of some parasites (Fig. 4). This finding indicates that the microvilli fused with each other, but the parasite was not completely covered with them.

The invasion process to the ileum epithelial cell

Cryptosporidium parvum zoites were located among the microvilli of host cells (Fig. 5). The microvilli of the host cell elongated along both sides of the zoite further than those of uninfected cells. The pellicle was separated from the host cell membrane at the attachment site (Fig. 6). The host cell membrane was observed under the pellicle and was continuous with both sides of the microvilli. A dense band was formed under the host cell membrane in the cytoplasm. In addition, a vacuole was formed in the cytoplasm at the base of the zoite. Figure 7 shows 2 zoites completely covered with the membrane derived from the microvilli. They thus occur in a parasitophorous vacuole formed by the microvillar membrane. Membranes surrounding zoites were composed of double layers. The microvilli between these 2 zoites were remarkably elongated. The pellicle was still separated from the host cell membranes at the attachment site (Fig. 8). The entire membrane around the zoite was ruthenium red-positive even after the zoite adhered to the host cell (Figs. 9, 10). The area between the host cell membrane and the dense band was also positive. The zoite was completely covered with the membrane derived from the microvilli, but the pellicle and host cell membrane were indistinguishable at the attachment site (Fig. 11). With ruthenium red staining, membranes surrounding the zoite were positive, but the attachment site at the base of the parasite became negative (Fig. 12). The pellicle and the host cell membrane were indistinct and ruthenium red-negative at the base of the zoites (Fig. 13). Additionally, the outer membrane of the microvilli was positive; in contrast, the inner membrane and the pellicle were negative in another region (Fig. 14) and even in the cross section of the zoite and the microvilli (Fig. 15). Pointed structures were observed



FIGURES 5–12. Transmission electron micrographs showing the invasion process of *C. parvum* into the host cell. Bars = 0.5 μm (5, 7, 9, 10, 11, and 12) and 0.1 μm (6 and 8). (**5**) *C. parvum* attaches to a host cell. The microvilli of the host cell elongate along both sides of the parasite (arrows). A dense band (DB) is found under the host cell membrane. (**6**) Attachment region between the host cell and parasite shown in Figure 5 at higher magnification. The pellicle (PE) is separated from the host cell membrane (HCM) at the base of the parasite. (**7**) *Cryptosporidium parvum* is completely covered with the membrane derived from the microvilli. Each parasite is in a parasitophorous vacuole (PV) formed by the microvilli. Membranes surrounding the parasites are composed of double layers. The microvilli (MV) between 2 parasites show remarkable elongation. DB, dense band. (**8**) Bottom region of the parasite shown in Figure 7 at higher magnification. The pellicle (PE) is still separated from



FIGURES 13–18. Transmission electron micrographs showing the invasion process of *C. parvum* into the host cell. Bars = 0.5 μm (13, 15, 17, and 18) and 0.1 μm (14 and 16). (**13**) The parasite is completely covered with the ruthenium red-positive membrane derived from the microvilli. Note that the base of the parasite is ruthenium red-negative. (**14**) Upper portion shown in Figure 13 at higher magnification. The outer membrane of the microvilli (OM) shows a positive reaction of ruthenium red staining, but the inner membrane (IM) and the pellicle (PE) show a negative one. (**15**) A cross section of the parasite and microvilli (the same stage as Fig. 13). (**16**) A portion shown in Figure 15 at higher magnification. The ruthenium red-positive areas are dot-like and are observed between OM and IM (arrow). (**17**) The fusion of the microvilli is incomplete at the upper part of the parasite (arrowhead). Note that the parasite pellicle attaching to the host cell membrane (HCM) shows complexity (arrow). A dense band (DB) is formed in the host cell cytoplasm. (**18**) The parasite is completely covered with a double-layered membrane derived from the microvilli. The parasite pellicle becomes more complex and forms a feeder organelle (*) at the base of the parasite. The feeder organelle adheres to the host cell membrane (HCM). The margin of the adhesion area is more electron-dense (arrow) than the other part.

←

the host cell membrane (HCM). (**9**) The membranes surrounding the parasite are ruthenium red-positive (dark-colored region) as well as are the microvilli of epithelial cells. (**10**) Parasite pellicle attaching to the host cell membrane (HCM) at the base of the parasite by ruthenium red staining. The attachment area is ruthenium red-positive (arrow) in the membrane completely surrounding the parasite. DB, dense band. (**11**) The parasite is completely covered with a double-layered membrane derived from the microvilli. The pellicle and host cell membrane are not clearly observed (arrow) at the base of the parasite. DB, dense band. (**12**) Membranes surrounding the parasite are ruthenium red-positive, as in Figure 9, but the attachment area is ruthenium red-negative (arrow).

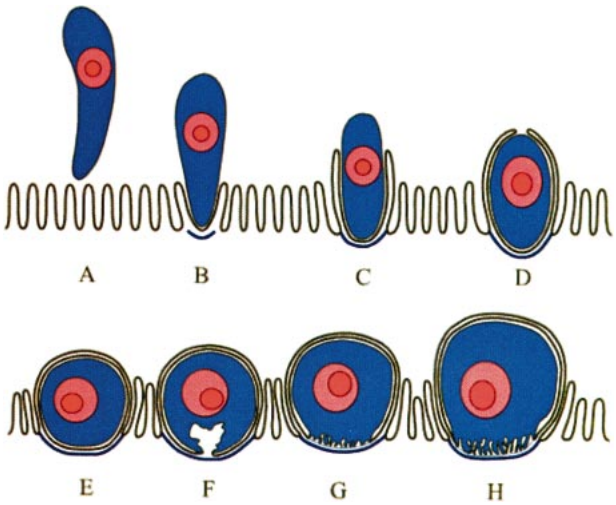


FIGURE 19. Schematic diagram of host cell invasion by *C. parvum*. First, a free parasite enters the host intestinal lumen (**A**) and then attaches to the surface of the epithelium among the microvilli (**B**). Subsequently, the microvilli elongate along the surface of the parasite (**C**, **D**), and a dense band is formed within the host cell cytoplasm. After the parasite is completely covered with microvilli (**E**), the parasite pellicle and host cell membrane become unclear (**F**). Furthermore, a 'feeder' organelle forms at the base of the parasite (**G**, **H**).

between the outer and inner membranes, and some ruthenium red-positive parts were present (Fig. 16). The pellicle became complicated at the base of the zoite, although the fusion of the microvilli was incomplete at the upper part of the zoite (Fig. 17). The host cell membrane was found under the pellicle. A dense band was present in the host cell cytoplasm. The pellicle became more complicated and formed a 'feeder organelle' at the base of the zoite (Fig. 18). The 'feeder organelle' attached to the host cell membrane, and the margin of adhesion area showed high electron density.

Subsequently, the pellicle and host cell membrane began to fuse after the zoite was completely enveloped with microvilli in most cases, but the fusion had already begun before complete envelopment in others.

DISCUSSION

Diagrams of the invasion process of *C. parvum* into host cells are shown in Figure 19. First, the zoite attached among microvilli, and the microvilli then gradually elongated along the surface of the zoite (Fig. 19A–C). The microvilli along the invading zoite were thinner than those in the uninfected region. These findings suggest that the zoite invaded the host cell by pressing against the host cell membrane among the microvilli after it attached. The zoite then barely invaginated the host cell with the elongation of the microvilli and assumed a circular form (Fig. 19D). A dense band emerged under the host cell membrane at the attachment area between the zoite and the host cell. A parasitophorous vacuole was formed after the microvilli fused, and the zoite was completely covered as a result (Fig. 19E). However, we could not determine whether the elongation of the microvilli was caused by zoites or by host cells. Elliott and Clark (2000) reported that *C. parvum* appears to utilize f-actin as a structural component of the host–parasite junction complex and that f-actin may play more active roles in their invasion process. As they showed, the cytoskeleton molecules of the microvilli may be affected by parasites. However, this process has not been elucidated.

The vacuolation of the zoite cytoplasm was found from the early invasion of zoites into the host cell, leading to formation of a parasitophorous vacuole. Vacuolation has also been reported ultrastructurally in *Cryptosporidium* spp. (Vetterling et al., 1971; Current and Reese, 1986; Lumb et al., 1988; Ostrovska and Paperna, 1990). It is known that the vacuolation occurs as a result of secretion from the micronemes, rhoptries, and dense granules.

After attachment of the parasite pellicle and host cell membrane, both membranes became unclear and ruthenium red-negative. This suggests that intracellular parasitism was established because ruthenium red cannot normally enter the cells (Takada and Hirano, 1983). The ruthenium red-positive reaction shows that the membrane enveloping the zoites is derived from the microvilli. These results differ from the report by Chen et al. (1998) that the zoite is covered by an extension of the host cell membrane. One reason for the difference between their results and ours may be that their studies were conducted in vivo, using cultured human biliary epithelia.

Our results generally support the observations by Marcial and Madara (1986) regarding the invasion process in *Cryptosporidium* sp. However, their schematic diagram showed that the zoite attached to the microvilli and then invaded by pressing numerous microvilli. The ''moving junction'' shown by them was not evident in our observation. Moreover, Iseki (1979) reported that the electron-dense band was formed at the base of the microvillus; the dense band in the present study was similar to the one reported by Vetterling et al. (1971).

Effective disinfectant and drug therapy for *C. parvum* have not been completely developed (Petri, 2003). Following penetration, *C. parvum* is situated inside the host cell, but outside of the cytoplasm. In contrast, other coccidia, such as *Toxoplasma gondii* and *Eimeria* spp., invade the host cell's cytoplasm. If the details of the mechanism of entry by *C. parvum* zoites can be elucidated, a potent and effective therapy for infection might be developed.

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