Ultrastructure of *Cryptosporidium parvum* Found in the Small Intestine of Immunosuppressed Mice [t]

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=Abstract=The ultrastructure of various stages of *Cryptosporidium parvum* was observed by transmission electron microscopy. *C. parvum* infection was activated in the small intestine of Korean laboratory mice (ICR) by immunosuppression with prednisolone for 7 weeks. The oocyst discharge was confirmed by modified Kinyon's acid fast stain of fecal specimens. Various endogenous stages of parasites, *i. e.*, trophozoites, meronts, merozoites, and macrogametocytes, were observed in the middle part of the small intestine, as an extracytoplasmic but intracellular parasite of host mucosal epithelial cells. In trophozoites, a large nucleus with a prominent nucleolus was seen, and as they developed into meronts, endoplasmic reticulum appeared prominently in the cytoplasm. Two kinds of meronts, type I and type II, with eight and four merozoites respectively, were found. New merozoites were produced by nuclear division and external budding of the residual body of the meronts. The merozoites were lined with two unit membranes, unlike *C. muris* that has three membranes, and a nucleus was located near the posterior end. Mature merozoites had conoids, rhoptries and numerous micronemes; the characteristic structures of coccidian parasites. Macrogametocytes were largely vacuolated and "wall-forming body I" was recognized. Other sexual stages were difficult to recognize from our specimens. The present study confirmed that the *Cryptosporidium* found in the small intestine of Korean laboratory mice has a characteristic ultrastructure consistent with *C. parvum*.

Key Words: *Ultrastructure, Cryptosporidium parvum, Mouse, Immunosuppression*

INTRODUCTION

The genus *Cryptosporidium* is one of the coccidia of the suborder Eimeriorina, phylum Apicomplexa. The first description of this coccidian parasite was made by Tyzzer (1907), with *C. muris* in the gastric gland of laboratory mice. Another species, *C. parvum*, was later found in the small intestine of common mice (Tyzzer 1912). After this, more than twenty

species of *Cryptosporidium* were described in the literature according to the host in which the parasite was found. Reviewing these species, however, Levine (1984) concluded that only 4 species should be considered valid, and that in mammalian hosts, only *C. muris* was valid. But, Upton and Current (1985) disagreed with Levine's (1984) consolidation of mammalian species of *Cryptosporidium*, and argued that the two species infecting mice, *C. muris* (Tyzzer 1907) and *C. parvum* (Tyzzer 1912) are both morphologically and biologically distinct.

The taxonomy of *Cryptosporidium* spp. is becoming more important since humans are now well known to be a potential host for this parasite (Meisel *et al.* 1976; Nime *et al.* 1976; Fayer and Ungar 1986). At present crypto-sporidiosis is recognized as a life threatening disease of man, with overwhelming diarrhea and enterocolitis, especially in immuno-compromised patients such as AIDS (Fayer and Ungar 1986; Current 1989). At least one species, *C. parvum*, is recognized as infecting immuno-competent humans (Current 1989), and it should be ruled out that many others, of animal origin, could infect immunocompromised humans.

Cryptosporidium spp. are usually found as 'small particles' attached to the brush border of host mucosal epithelia, and each species has its characteristic morphology of trophozoites, meronts (schizonts) and merozoites, macro- and microgametocytes, zygotes, oocysts and sporozoites. However, the size of each stage is so minute, usually smaller than 7-8 μm, that ultrastructural studies are often necessitated to compare and diagnose the species. Thus, the ultrastructure of *Cryptosporidium* has been studied in *C. parvum* of mice (Hampton and Rosario 1966) and rats (Beier and Sidorenko 1990), *C. wrairi* of guinea pigs (Vetterling *et al.* 1971), *C. felis* of cats (Iseki 1979), *C. muris* of mice (Uni *et al.* 1987; Iseki *et al.* 1989; Rhee *et al.* 1991), *Cryptosporidium* of human origins (Bird and Smith 1980; Current and Reese 1986) and so on. From a study with *C. parvum*, Hampton and Rosario (1966) confirmed that

Cryptosporidium is an intracellular parasite, enveloped with thin membranes of host cell origin.

In Korea, Chai *et al.* (1990) have reported the presence of *C. parvum* in the small intestine of laboratory mice by immunosuppression with prednisolone, but its ultrastructure has not been described in detail. This report aims to confirm the species of *Cryptosporidium* found in Korean laboratory mice by an electron microscopic study and to provide some basic knowledge of the ultrastructure of *C. parvum*, which could be used as a reference for the diagnosis of possible human cryptosporidiosis in Korea.

MATERIALS AND METHODS

1. Activation of *C. parvum* by immunosuppression

Male ICR mice (Animal Center of Seoul National University, Seoul) weighing between 13 and 15 grams were used, and the procedure of Chai *et al.* (1990) was followed for the activation of *Cryptosporidium*. Briefly, while control mice were maintained on a regular 22.1% protein pelleted diet and tap water, test mice were injected with prednisolone 1.0 mg intramuscularly every other day for 7 weeks and placed on a regular protein diet and drinking water containing trimethoprim-sulfamethoxazole (1mg/ml) to inhibit the growth of bacteria. All of the control and test mice were checked for discharge of oocysts by modified Kinyon's acid fast stain of fecal samples on a weekly basis.

2. Ultrastructural observation

Mice shedding oocysts at 7 weeks after the start of immunosuppression were killed by cervical dislocation and small pieces of tissues were taken from the lower part of the jejunum. The samples were fixed in 2.5% glutaraldehyde and followed by post-fixation with 1% OsO$_4$ in 0.1 M phosphate buffer. The fixed tissues were washed, dehydrated, and embedded in Epon 812 resin. Ultra-thin sections from the Epon block were stained with uranyl acetate and lead citrate, and examined under a Zeol 100 CX2
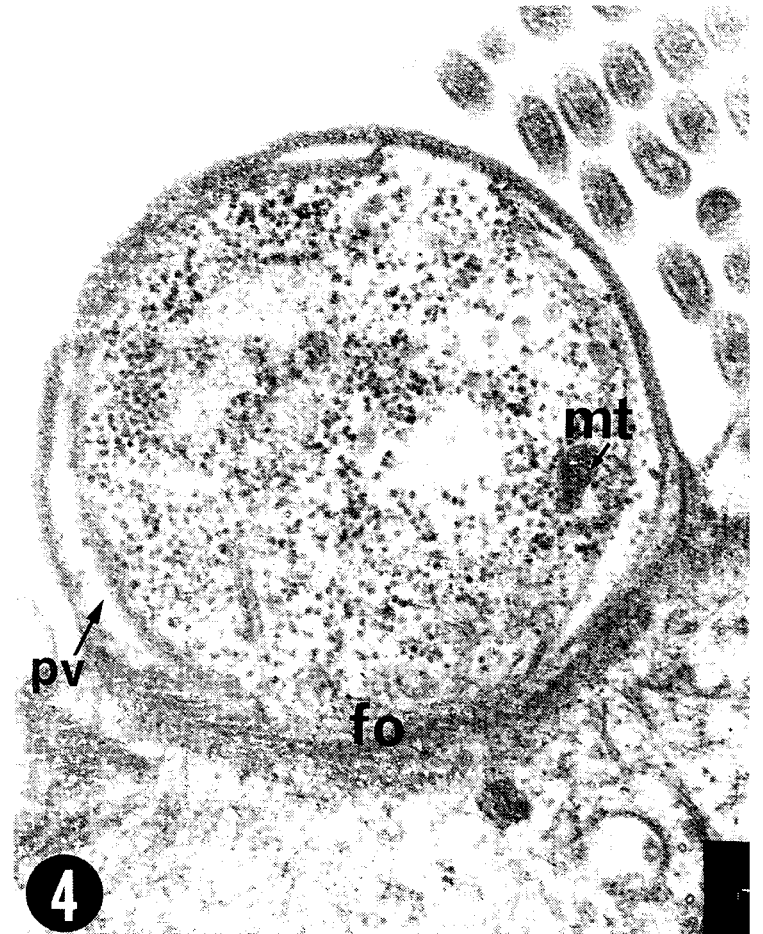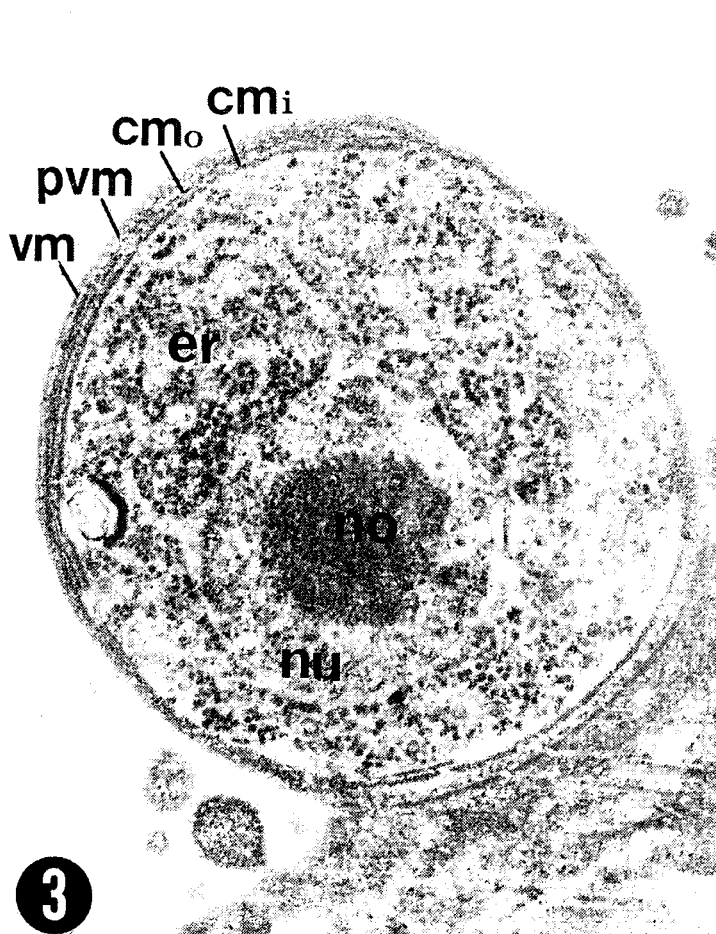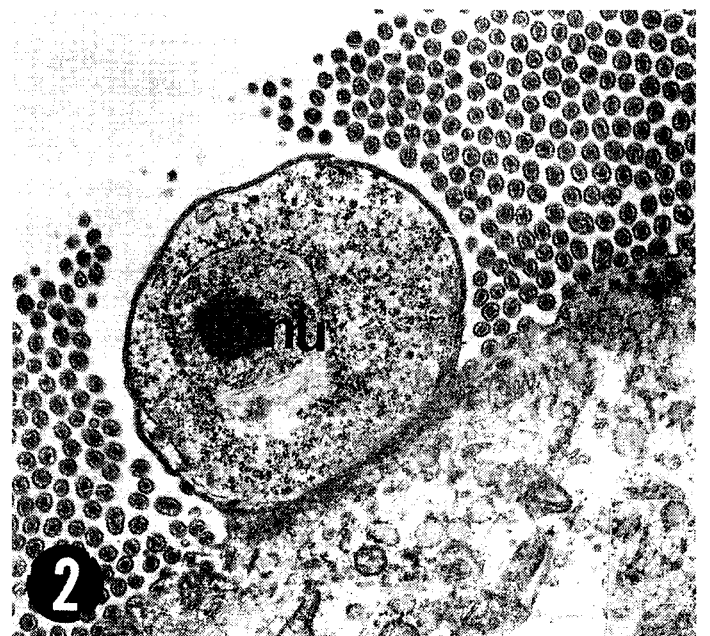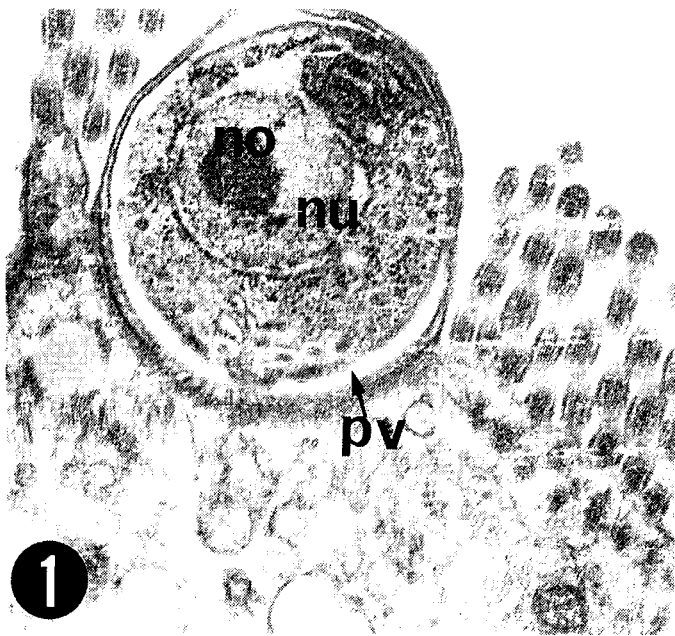
Figs. 1-2. Early trophozoites having a large nucleus (nu) and nucleolus (no). Note the parasitophorous vacuole (pv) by which the trophozoite is enveloped. x 10,000

Fig. 3. A developing trophozoite with a large nucleolus (no) occupying most of the nucleus (nu) and a large rough endoplasmic reticulum (er). The pellicle includes four unit membranes, the outer two from the host microvillus and the inner two from the parasite. The inner membrane of the pellicle has been resorbed at the lower end portion of the parasite. cmi: inner cytoplasmic membrane of parasite origin, cmo: outer cytoplasmic membrane of parasite origin, pvm: parasitophorous vacuole membrane, vm: outer villous membrane. x 14,000

Fig. 4. Another developing trophozoite surrounded by a parasitophorous vacuole (pv) and containing mitochondria (mt) and ribosomes scattered throughout the cytoplasm. The attachment zone between the host and the parasite became wrinkled to form the 'feeder organelle' (fo). x 14,000

transmission electron microscope.

RESULTS

The populations of early, developing, and mature trophozoites were most abundant among all life cycle stages. The trophozoites were generally round, each with a conspicuous nucleus and a nucleolus (Figs. 1, 2 & 3). It was shown that the invasive stages (sporozoites or merozoites) were, after their invasion into host epithelial cells, transformed into an ovoid or round form (trophozoites) surrounded by a parasitophorous vacuole (Fig. 1). All of the organelles of the invasive stages were seen to have been resorbed except for their nucleus, pellicle and cytoplasmic ribosomes. These were the earliest trophozoites (Fig. 1).

Most of the developing trophozoites were surrounded by a pellicle composed of four unit membranes, the outer two from host microvilli and the inner two from the parasite themselves (Fig. 3). The four unit membranes became two as the trophozoites became mature. The attachment organ, named the 'feeder organelle', was composed of many membranous folds continuous to the pellicle of the parasite, and was well developed in an end region of developing or mature trophozoites (Fig. 4). Within the cytoplasm there were rough endoplasmic reticula, mitochondria, electron dense granules just like fragments of micronemes, and many ribosomes (Figs. 3 & 4).

Mature trophozoites were found to develop into type I meronts first, and as the meronts became mature, endoplasmic reticulum and vacuoles were well developed in the cytoplasm (Fig. 5). Merozoite formation was initiated by nuclear division of the meronts (Fig. 6), and then by external budding of their cytoplasm and rhoptries formation (Figs. 7 & 8). These new merozoites developed again into new trophozoites and then into type I (Fig. 9) or type II meronts (Fig. 10).

The number of merozoites were, in most cases, 8 in type I and 4 in type II meronts (Figs. 9 & 10). The residual body of the meronts was lined with one parasite membrane. The anterior half of mature merozoites was filled with numerous micronemes and rhoptries, and near the anterior end conoids were also observed. A nucleus was present near the posterior end of each merozoite and a rough endoplasmic reticulum was located in front of the nucleus (Figs. 11 & 12). Characteristically at the anterior end of each merozoite a flask shaped pouch, half-filled with small granules, was present (Fig. 12). In the cytoplasm of merozoites numerous ribosomes were also observed (Fig. 12). Refractile bodies or polysaccharide granules were not found in the cytoplasm.

The cytoplasm of the macrogametocytes was largely vacuolated and featured with prominent, electron dense, polysaccharide granules (Fig. 13). Another kind of electron dense granules, smaller-sized and round, probably the "wall forming body I" (Current and Reese 1986), were also characteristically seen (Fig. 13). It was difficult to find other stages of the gametogony cycle such as microgametocytes and zygotes. Oocysts were not identified from the observed specimens.

These ultrastructural findings, together with the oocyst size of 4-6 μm and the habitat of the parasite (small intestine), were all compatible with previous descriptions of C. parvum.

DISCUSSION

Concerned with the fine structure of Cryptosporidium, Hampton and Rosario (1966) first described the ultrastructure of C. parvum in the ileum of laboratory mice, and confirmed that Cryptosporidium is an intracellular parasite. After this, the ultrastructure of other species such as C. wrairi (Vetterling et al. 1971), C. felis (Iseki 1979) and C. muris (Rhee et al. 1991) were described.

At present, it is generally accepted that many species of Cryptosporidium reported are host-specific parasites. However, morphological difference among the species has seldom been described, except in the size of oocysts or some other stages. In this study, we noticed some
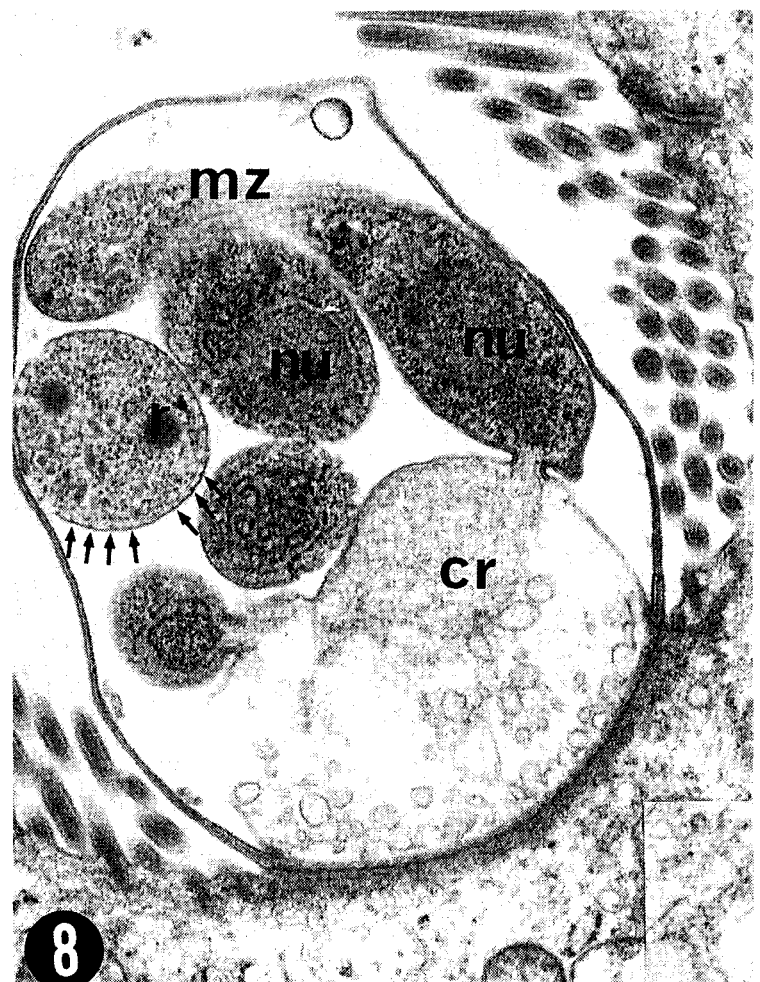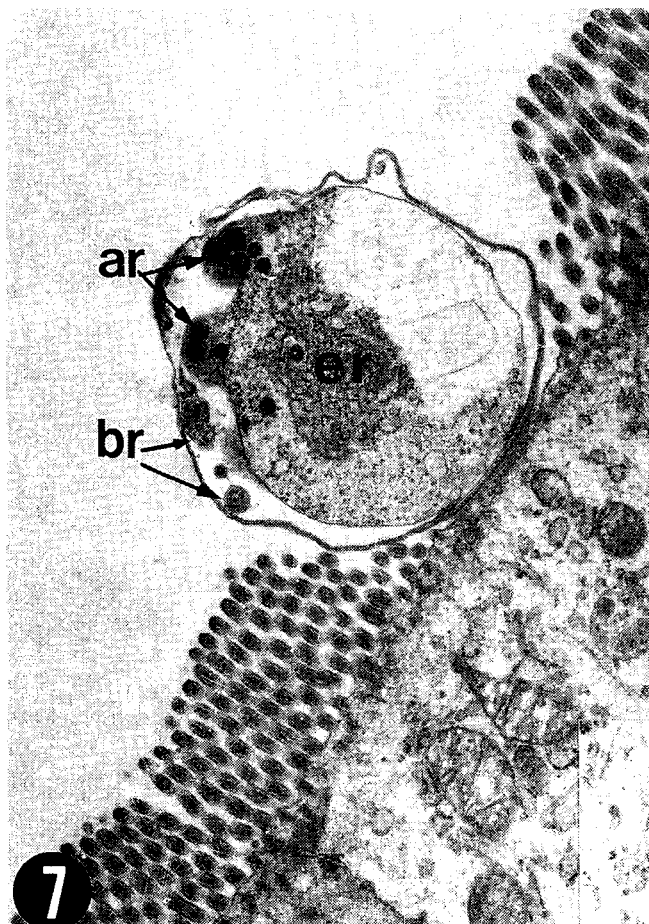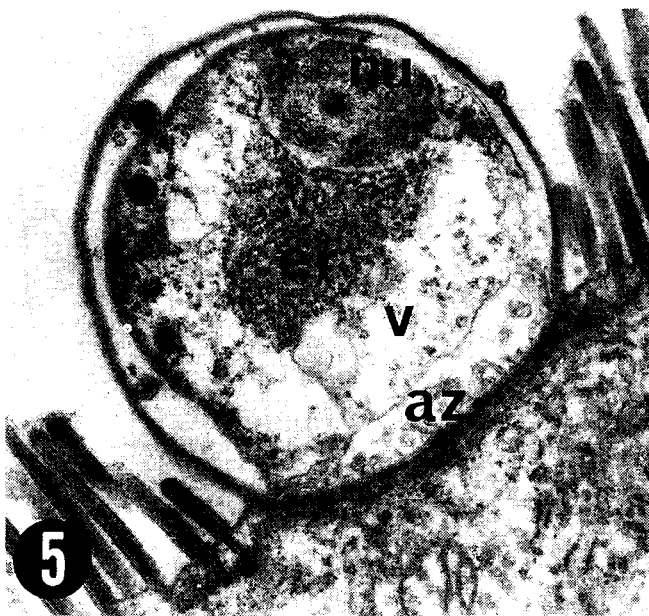
Fig. 5. An early meront with one nucleus (nu). The cisternal rough endoplasmic reticulum (er) is well developed. Several dense granules have been formed. Approximately half of the cytoplasm is filled with vacuoles (v). az: attachment zone. x 12,000

Fig. 6. Another early meront showing nuclear division (nu). The endoplasmic reticulum (er) and vacuole (v) are prominent. x 12,000

Fig. 7. A meront in the phase of external budding. The budding merozoites include anlagens (ar) of rhoptries and micronemes. Single membrane-bound blebs (br) are found in the parasito/phorous vacuole. Endoplasmic reticulum (er) is seen in the center of the parasite. x 10,000

Fig. 8. A mature meront, presumably type I, showing at least 5 merozoites (mz), each of which has one nucleus (nu) and rhoptries (r) and is bounded by double unit membranes. Merozoite budding from the cytoplasmic residuum (cr) is seen to occur from anterior to posterior direction. Electron dense fibril-like structures are seen on the inner surface of the outer unit membrane (arrows). x 14,000
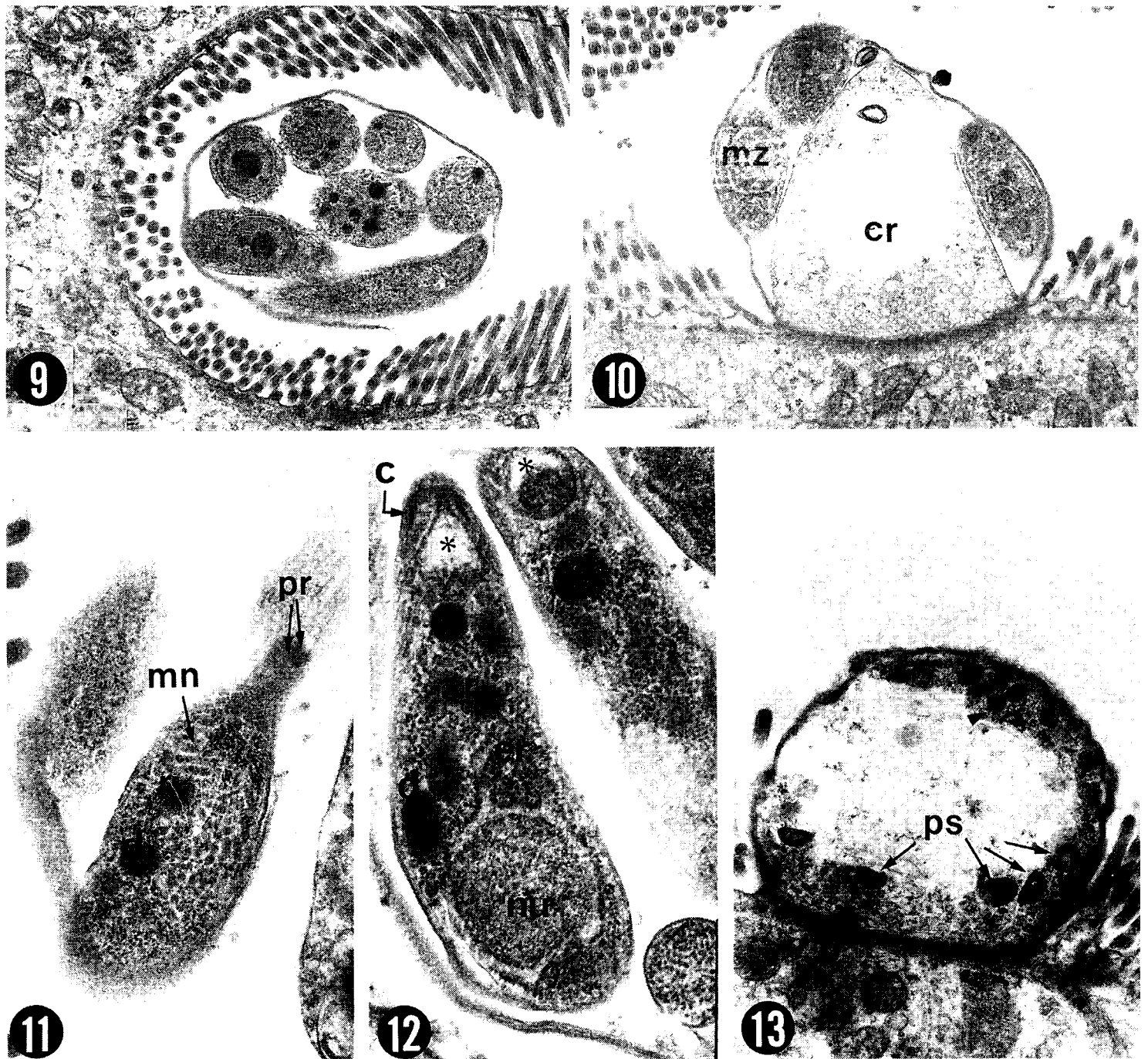
Fig. 9. Another mature type I meront. Seven merozoites are visible in this section. Merozoites have a nucleolated nucleus with dense granules, rough endoplasmic reticulum, and micronemes. x 10,000

Fig. 10. A mature type II meront showing new merozoites (mz) and its cytoplasmic residuum (cr). x 12,000

Fig. 11. An isolated merozoite, showing the dense granule (d), rhoptry (r), microneme (mn), and polar ring (pr). x 14,000

Fig. 12. Mature merozoites in a type I meront showing conoids (c), rhoptries (r), dense granules (d), a rough endoplasmic reticulum (er) and a nucleus (nu). Note that the nucleus is located near the posterior end and the endoplasmic reticulum is in front of the nucleus. At the anterior end portion an electron pale structure (*) thought to be a cytostome or an opening of the rhoptry is present. x 16,000

Fig. 13. A macrogametocyte with several polysaccharide granules (ps). The cytoplasm is largely vacuolated and contains several electron dense granules thought to be the "wall-forming body I" (arrow heads). x 12,000

points of difference between *C. parvum* and other species, especially in the merozoite stage.

The locations of the nucleus and endoplasmic reticulum between the merozoites of *C. felis* and *C. parvum* were different. The nucleus of *C. felis* was located in the middle portion of the body (Iseki 1979), whereas in the case of *C. parvum* it was situated near the posterior end portion. Rough endoplasmic reticula were located in the posterior region in *C. felis*, whereas in *C. parvum* they were mostly in the middle portion. Also no typical forms of mitochondria, micropores, polysaccharide granules, refractile granules and subpellicular fibrils, as described in *C. wrairi* (Vetterling *et al.* 1971), were found in merozoites of *C. parvum* in this study.

The constitution of the surrounding pellicle in merozoites of *C. parvum* was also different from that of other species. The merozoites of *C. felis* and C. muris were surrounded by a pellicle of three unit membranes, and electron dense fibril-like structures were present on the outer surface of the middle unit membrane (Iseki 1979; Rhee *et al.* 1991). However, the merozoites of *C. parvum* were surrounded by a pellicle of two unit membranes, and electron dense fibril-like structures were present on the inner surface of the outer unit membrane.

No special difference was noted in the present study between the type I and type II merozoites of *C. parvum*, except in the presence of electron dense fibril-like structures on the inner side of the outer unit membrane in the former. Although such a structure was not identified in type II merozoites, no one could say that they were never present. Further studies should be performed to verify this point.

Speculations on the functions of fine structures of *Cryptosporidium* or other coccidian protozoa have been made by various workers (Aikawa and Sterling 1974; Current and Reese 1986). A pouch at the anterior end of merozoites of *C. parvum*, together with electron-dense granules, was thought to be a cytostome or opening of the rhoptry. The number of rhoptries in the sporozoites and merozoites of

some species of Eimeria was reported as two (Aikawa and Sterling 1974), but in the case of *C. parvum* the number was more than two. The apical complex such as the conoid, microneme and rhoptries was considered to participate in the invasion process of coccidian parasites into host cells, and as a result to form an attachment zone between the parasite and the host (Current and Reese 1986).

The merozoites of *C. parvum* pinched off the cytoplasmic residuum of meronts by infolding of the double membrane, leaving a single membrane enclosing the residuum between the newly forming merozoites. Budding appeared to take place from the anterior body to the posterior one as described by Vettering *et al.* (1971) in *C. wrairi*.

Microgametocytes, zygotes and oocysts, and even macrogametocytes, were difficult to find in this study. However, since the mice examined were those shedding the oocysts, it is not likely that the sexual stages were almost stopped at 7 weeks after the start of immunosuppression. According to Iseki *et al.* (1989), the fecal discharge of *C. muris* oocysts in mice after an oral administration of oocysts appeared from 5-6 days and continued individually to 34-75 days after the infection. Similarly, Rhee *et al.* (1991) observed the fecal discharge of *C. muris* oocysts in mice from 5-6 days to 66-70 days after an oral infection. It was also reported in congenitally athymic nude mice that the oocyst discharge of *Cryptosporidium* sp. (calf origin) was continued at least for 8 weeks of the observation period after an oral infection (Heine *et al.* 1984).

It has not been studied how long the oocysts of *C. parvum* could be produced in immunosuppressed mice. Chai *et al.* (1990) and the present authors observed that the oocysts of *C. parvum* were detectable from the feces of mice at least for 7 weeks of the immunosuppression period. However, it is strongly suggested that at 7 weeks after immunosuppression the oocyst discharge might have been more or less reduced compared with earlier periods of immunosuppression. Dis-

covery of no oocysts from the gut epithelia in human cases of *Cryptosporidium* sp. (presumably *C. parvum*) infection (Bird and Smith 1980) might have been due to the chronicity of their infection. In other respects, short duration of oocyst accumulation or stay in the gut might be a characteristic feature of *C. parvum*. Anyhow, it is interesting to note that, even if sexual stages were suppressed, the asexual cycle was actively maintained up to 7 weeks in immunosuppressed mice. Further studies will verify how long the asexual and sexual stages could be continued in *C. parvum*.

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