Infection kinetics and developmental biology of *Cryptosporidium muris* (strain MCR) in Korean native kids and Corriedale lambs

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Abstract: A total of nine Korean native kids and two Corriedale lambs, 1-20 days old, were each inoculated per os with a single dose of 2×10^7 oocysts of *Cryptosporidium muris* (strain MCR) originated from mice to elucidate the kinetics and developmental stages of the coccidium in small ruminants. Irrespective of host's age, the prepatent period for both animals ranged from 19 to 35 days (28.1 days, on the average) and the patent period 16-85 days (47.8 days), and the total oocyst outputs showed enormous differences. Infection with greater numbers of oocyst outputs was not ordinarily established by transmission experiments. Oocysts discharged from the kids retained their infectivity by the mouse titration method. The immunogenicity of the coccidium and oocyst reproduction were proven by challenge infection and administration of prednisolone acetate, respectively. All the developmental stages of the coccidium in parasitophorous vacuoles were found by transmission electron microscopy in the pits of the gastric glands of a kid inoculated with oocysts and then necropsied on day 44 postinoculation. It indicated the full course of the host-parasite relationship in kids and lambs as well as mice.

Key words: *Cryptosporidium muris* (strain MCR), kid, lamb, oocyst production, endogenous development

INTRODUCTION

Since *Cryptosporidium muris* was first discovered by Tyzzer (1907) as a protozoan parasite of mouse stomach, many investigators have discovered oocysts of the protozoon in fecal samples of cattle world-widely (Upton and Current, 1985; Anderson, 1990 & 1992; Rhee *et al.*, 1991a; Nakai *et al.*, 1996). The parasite had not been reported for over 70 years since

its initial identification.

The majority of cryptosporidiosis in man and animals have been documented as intestinal cryptosporidiosis by *C. parvum*. Infection with *C. muris* in cattle may be asymptomatic or associated with transient symptom, therefore one has no concern for relating to the impact of the parasite on it's hosts. Recently, *C. muris* was rediscovered in the abomasum of cattle that had failed to eat well and failed to gain body weight optimally (Anderson, 1987). Thereafter, Esteban and Anderson (1995) reported that cows shedding *C. muris* oocysts produced significantly less milk (approximately 3.2 kg/day) and that mean daily milk production was significantly associated with

shedding status of oocysts. The protozoon resides in the peptic glands of the abomasum, causing dilation of the glands and mucosal hypertrophy. Microvilli of gland epithelial cells are destroyed at attachment sites of the parasite. Plasma pepsinogen concentrations are twice as high in parasitized than in non-parasitized animals (Anderson, 1987 & 1989).

Even though cows shedding *C. muris* oocysts produced significantly less milk with decreased growth rate, no reports, so far, have been published on the kinetics in details of *C. muris* infection in ruminants excepting fragmentary delineations (Anderson, 1987 & 1988). It is reasonable to use calf as an experimental animal to study the basic relationship of low productivity in cattle attributed to the protozoon. However, calf is not suitable to feeding and maintaining due to its large size in addition to low establishment (success rate) of transmission experiment (Anderson, 1988). Therefore, kids and lambs were selected as an animal model instead of calf. This has been examined by assessing the life cycle comprising of oocyst production and entity of developmental stages of the parasite in the abomasum of ruminants throughout the transmission experiments. The immunogenicity of the coccidium and oocyst reproduction were observed to elucidate the relationship between abomasal cryptosporidiosis and the protozoon.

MATERIALS AND METHODS

Animals used for the transmission experiments were Korean native kids and Corriedale lambs delivered from their dams free from oocyst discharge. One- to twenty-day-old nine kids and two lambs were inoculated per os with a single dose of 2×10^7 oocysts of *C. muris* (strain MCR) originated from mice, as previously described (Rhee *et al.*, 1995). Oocysts have been maintained in our laboratory since 1990. Experimental animals were separately housed in wire-floored cages with concentrates, roughage and fresh water available *ad libitum*. The cages were placed on trays containing 5 mm depth of water to keep the feces wet.

To determine the infectivity of the oocysts discharged from the kids to mice, 10 SPF ICR mice, 3-week-old, were each inoculated orally with a single dose of 2×10^6 oocysts originated from the kids.

While, three days after oocyst shedding ceased, 2 kids that had shed small numbers of oocysts were intramuscularly injected with 55 mg prednisolone acetate daily for 14 days to reproduce oocysts. After complete shedding of oocysts, a lamb and a kid that had shed a large number of oocysts were each challenged orally with a single dose of 2×10^7 oocysts generated from the lamb or the kid to observe the immunogenicity of the parasite. To compare morphological dimension of oocysts in relation to their origin, 50 oocysts from each origin were measured by an ocular micrometer.

Following inoculations, the procedures used for the feeding of the mice and the examination of fecal samples of the ruminants and mice were subjected to those described previously by Rhee *et al.* (1995).

To observe the developmental stages of the parasite, a 2-week-old kid inoculated orally with a single dose of 2×10^7 oocysts was necropsied on day 44 postinoculation (PI), when discharged 6×10^7 oocysts/day. The abomasal segments about 0.5 cm in each length were removed from peptic glandular region (spiral fold), fixed in 10% neutral formalin, and submitted to routine histologic processing. Paraffin-embedded sections were stained with hematoxylin and eosin, and PAS stain. In addition, the abomasal segments were fixed in Karnousky fixative (pH 7.4). The specimens were then washed in 0.1 M cacodylate buffer, postfixed in 2% sodium tetroxide solution in cacodylate buffer, and dehydrated in an ascending ethanol series. They were embedded in epoxy resin and sectioned with a diamond knife on a ultramicrotome. The ultrathin sections were stained with 20% uranyl acetate and examined with a JEM 1010 transmission electron microscope.

RESULTS

Discharge pattern of oocyst in feces
The pattern of oocyst shedding in *C. muris*

infection in 9 kids and 2 lambs is depicted in Table 1. Each animal began to discharg oocysts on the 19th and 35th days (the 28.1th day, on the average) PI depending on the cases. The total oocyst outputs were different among the individual kids. The maximum oocyst counts (500-600 $\times$ 10^6) were recorded from three kids (Case Nos. 1, 2 and 11), while a medium numbers of oocysts (10-30 $\times$ 10^6) were counted from another three kids (Case Nos. 3, 4 and 5). However, the remaining three kids (Case Nos. 6, 7 and 8) discharged only a few oocysts in their fecal samples. The lambs also showed different oocyst outputs. As many as 304 $\times$ 10^6 oocysts were recovered from one lamb (Case No. 9), while the other (Case No. 10) shed small numbers of oocysts. The patent periods for all animals ranged from 34 to 85 days, excepting a patency of 16 days in a lamb. It appears that the kids show longer patency than the lambs. In the first 2 cases of kids with greater numbers of oocyst shedding on comparable level, peak oocyst production with an undulating output occurred on the 78th and 112th days PI and declined rapidly thereafter until the oocysts were no longer

present (Fig. 1). In most cases of the remainder, oocysts were seldom detected about 60 days PI using a Fuchus-Rosenthal hemocytometer, however, qualitative determination by fecal flotation and Kinyoun's modified acid-fast staining methods detected low levels of oocyst shedding up to about 70 days PI. The kids with a moderate oocyst output revealed some degree of undulating fashion of oocyst production over a period from 25th to 60th days PI.

All the mice inoculated with oocysts derived from kids excreted numerous oocysts (1 $\times$ 10^7/head/day in peak oocyst production period). In this occasion, the coccidium had the prepatent period of 9-11 days and the patent period of 60-69 days. Six days following the first prednisolone treatment, 2 kids (Case Nos. 6 and 8) that had shed small numbers of oocysts discharged a few uncountable oocysts by 7 days post-unmedication. In challenge infection, oocysts were not detected in the feces of a lamb (Case No. 9) and a kid (Case No. 3) each inoculated primarily with 2 $\times$ 10^7 oocysts at any time. As shown in Table 2, morphological dimension of oocysts from

Table 1. The length of the prepatent and patent periods and the total oocyst excretion of ruminants inoculated with 2 $\times$ 10^7 *Cryptosporidium muris* (strain MCR) oocysts

Case No.	Host	Age (days)	Prepatent period (days)	Patent period (days)	Total oocyst output ($\times$ 10^6)	Remarks
1	goat[a]	20	32	83	594.8	
2	goat	20	30	85	613.5	
3	goat	14	24	45	29.9	challenge
4	goat	14	26	43	13.7	
5	goat	7	19	34	9.7	
6	goat	3	34	45	small numbers[d]	reappearance
7	goat	3	27	44	small numbers	
8	goat	3	35	44	small numbers	reappearance
9	sheep[b]	1	28	38	304.3	challenge
10	sheep	1	35	16	small numbers	
11[e]	goat	15	19	> 25	> 564.5	victimization
mean		9.2	28.1	47.8	156.6	
± SD		7.5	5.8	21.1	253.6	
CV (%)[c]		82.0	20.6	44.1	161.9	

[a]Korean native kid. [b]Corriedale lamb. [c]Coefficient of variation: Standard deviation/mean $\times$ 100. [d]The number of oocyst shedding per day was too low to count with a Fuchus-Rosenthal hemocytometer. [e]The partial data of case number 11 were subtracted from arithmetic treatment due to interruption of observation by victimization for LM and TEM.
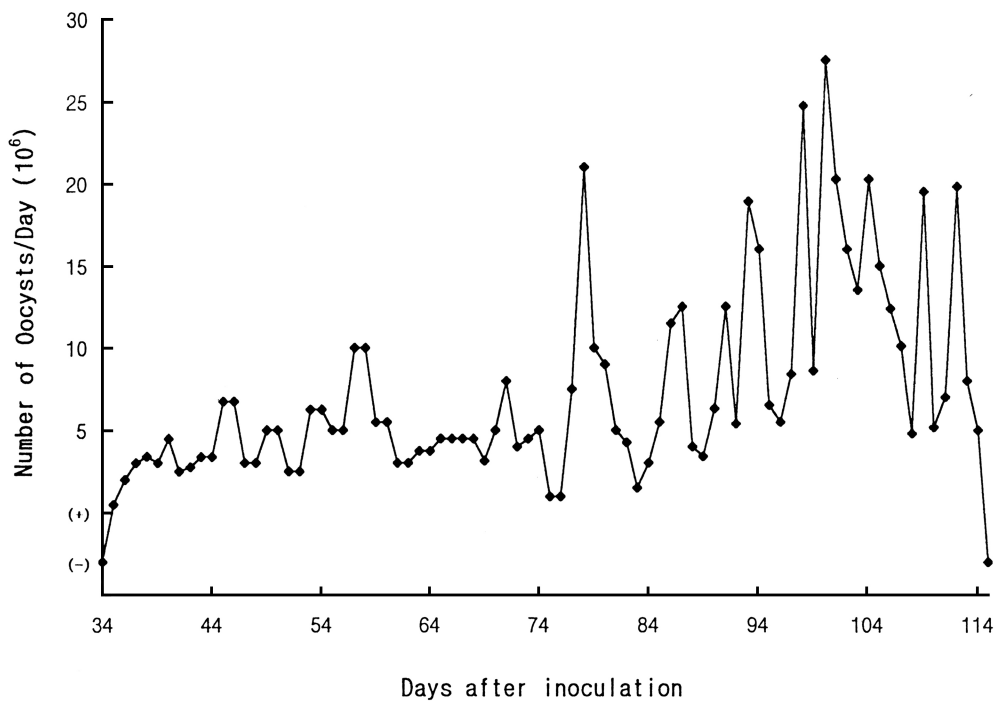
Fig. 1. Mean daily oocyst excretion of two kids each inoculated orally with 2×10^7 oocysts of *C. muris* (strain MCR) at 20 days of age. (+) and (−) indicate that less than 10^3 oocysts were detected and oocysts were not detected, respectively.

mouse to goat model system was similar to that from goat to mouse model, whereas oocysts in both systems were somewhat smaller than those of strain RN 66 and MCR of *C. muris*.

Structure of developmental stages

On light microscopy, cryptosporidia were seen in the abomasal peptic glands of an infected kid. Most glands were found infected, and various developmental stages were observed from the apex to the orifice of the glands (Fig. 2). Under transmission electron microscopy, all developmental stages in the parasitophorous vacuoles (PV) were found in the pits of the gastric glands. The endogenous development of the parasite occurred in the microvilli of the surface mucus cells of the gastric glands. A projection of the parasite, which was called a "knob like projection" by Tyzzer (1910), was seen at the attachment site to the host cells. The outer aspect of the projection was surrounded by a thick, filamentous process of the host cell.

The round trophozoite; 5.1 (5.0-5.2) × 4.6 (4.4-4.8) μm in size, had a relatively large nucleus with a prominent nucleolus. The trophozoite, which was covered by a pellicle with a double membrane, was enclosed by the inner parasitophorous vacuolar membrane (PVM) and the outer villous membrane (VM) originating from the microvillus of the host cell. Membranous lamellae of a feeder organelle occupied the inner part of the projection of the trophozoite. The feeder organelle appeared to be in contact with the cytoplasmic membrane of the coccidium at an annular ring. The annular ring was seen at a junction consisting of the cytoplasmic membrane of the coccidium and the PVM. This junctional complex appeared to anchor the periphery of the feeder organelle firmly to the base of the PV (Fig. 3). A dense band was seen at the base of the microvillous area where the parasite had invaded. An indented border separated the projection from the filamentous process of the host cell (Fig. 4).

The trophozoite developed into the meront,

Table 2. Comparison of dimension of oocysts in relation to their origin

| Strains or origins	Length (μm)	Width (μm)
RN 66	8.42 ± 0.41	6.54 ± 0.37
MCR	7.80 ± 0.56	5.89 ± 0.36
Mouse/goat system	7.70 ± 0.18	5.22 ± 0.19
Goat/mouse system	7.63 ± 0.22	5.20 ± 0.14

Each value represents the mean of 50 determinations with the standard deviations.

in which eight merozoites were formed following three nuclear division. Various developmental stages of the meront measured 6.2 (5.4-6.8) × 5.4 (5.2-5.9) μm. The meront contained developing merozoites, a mass of residual body and a well developed feeder organelle in the PV (Fig. 5). As merogony proceeded, the residual body of the meront was reduced. The fully developed merozoites, measuring 8.2 to 9.6 by 1.2 μm, in the PV were banana-shaped, and covered with a three unit membranous pellicle.

The macrogametes measured 7.1 (6.8-7.4) × 6.3 (5.9-6.8) μm, and the feeder organelle was well developed. As the parasite developed, amylopectin bodies were distributed throughout the cytoplasm, and wall-forming bodies containing material of different electron densities became more numerous and distributed in the peripheral cytoplasm. The parasite became round at the mature stage (Fig. 6).

The microgametocyte was identified by the formation of microgametes and a large residual body. The microgametes were formed around the residual body. The parasites were produced by budding from the large microgametocyte: 16 microgametes were formed by microgametogenesis. The anterior part of the parasites was slightly expanded and a compact nucleus was surrounded by microtubules. Each parasite had a large nucleus and microtubules (arrows) paralleled to the long axis in its cytoplasm (Fig. 7).

The oocyst, measuring 8.6 (7.8-9.0) × 6.5 (5.8-7.0) μm, was easily distinguished by a conspicuous oocyst wall composed of a double- or four-layered membranes. Sporogony took place in the PV, resulting in the formation of four sporozoites each of which had a nucleus,
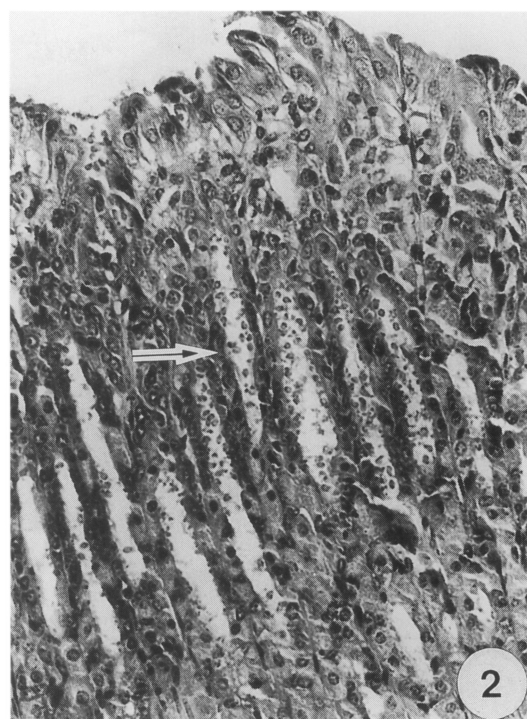

Fig. 2. Most abomasal peptic glands (arrow) of an infected kid are filled with large numbers of various cryptosporidial forms, × 350, HE.

electron dense bodies and vacuoles in the cytoplasm. The mature sporozoite was banana-shaped, and its cross-section showed a round appearance (Fig. 8).

DISCUSSION

Although the contention for taxonomy is continued until current, *C. muris* and *C. parvum* are valid, and the organism discovered in the abomasum of cattle is *C. muris* because ultrastructure of developmental stages, location of parasitism and the prepatent and patent periods differ from each other enormously.

Age-dependent resistance to *Cryptosporidium* spp. or susceptibility to cryptosporidiosis of animals varies with the species of protozoa as well as age of the host. Lambs became resistant to experimental infection with *C. parvum* isolated from calves by about 15-20 days of age, and those infected at 30 days of age became infected with the organism and shed oocysts for a brief periods (1-2 days), but
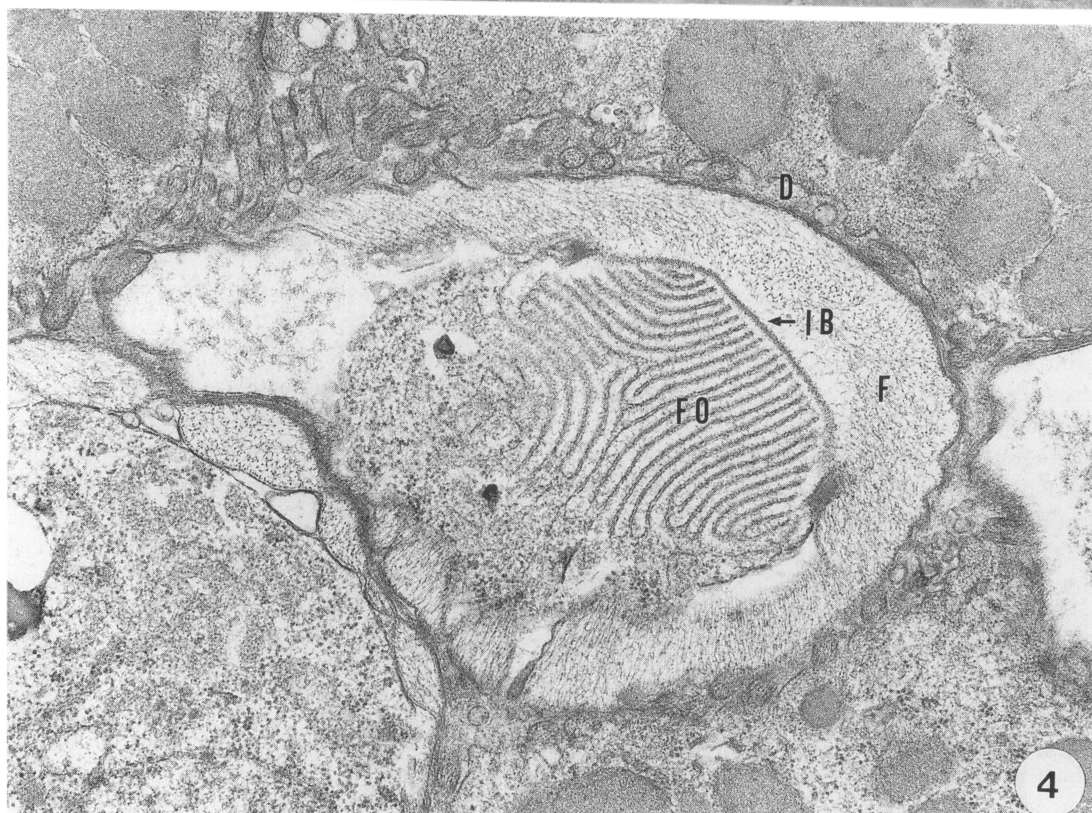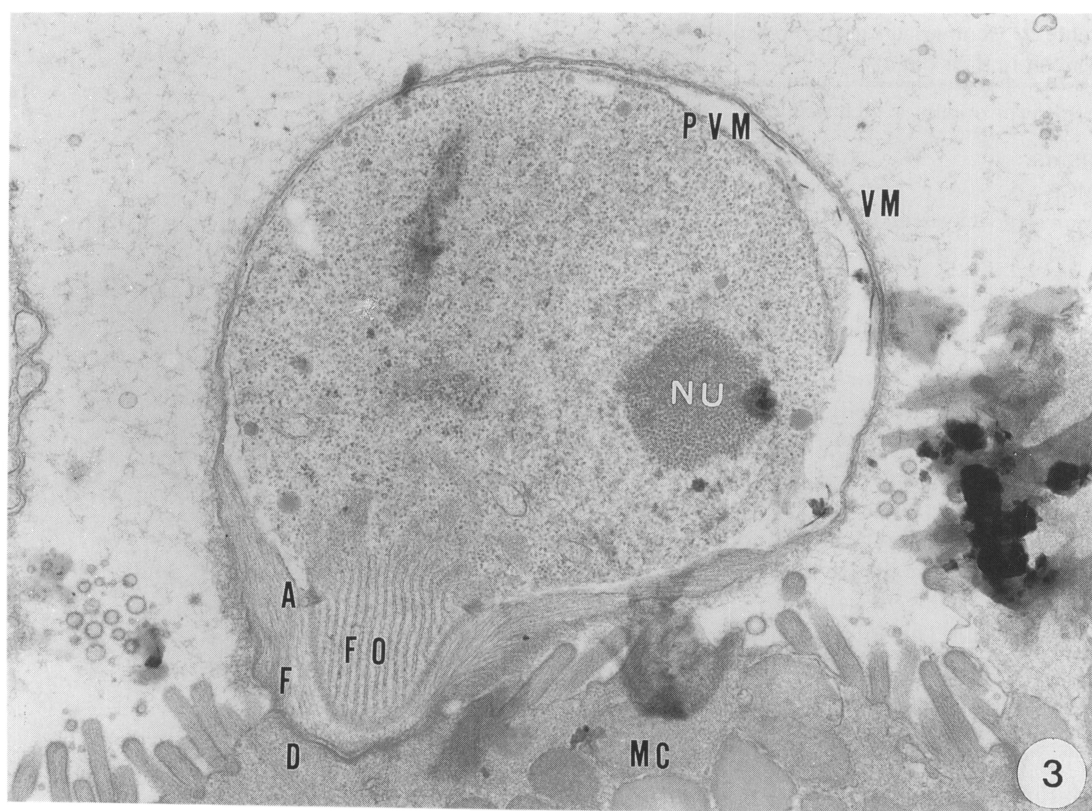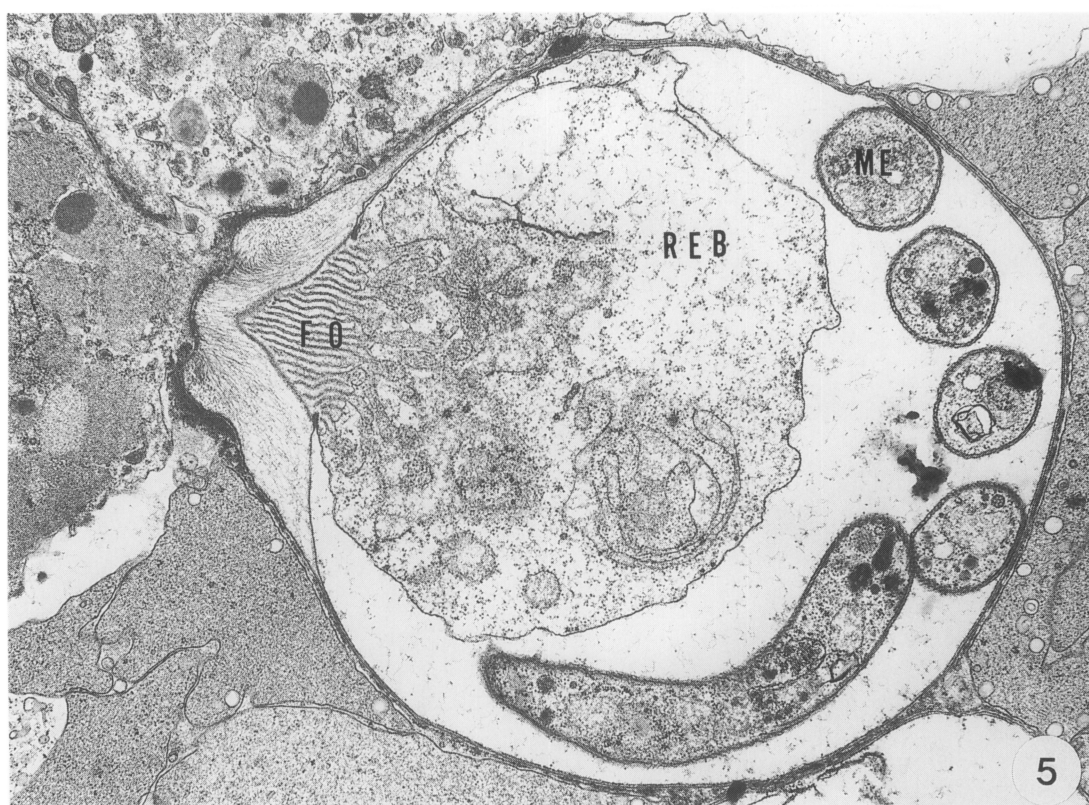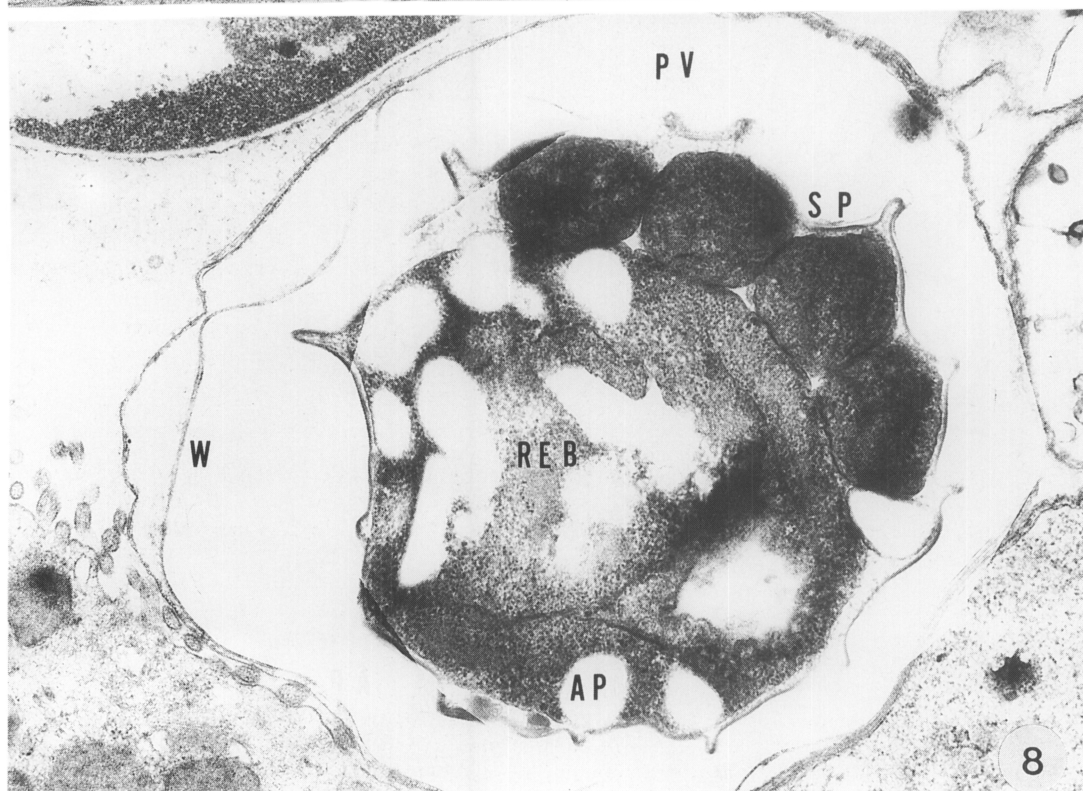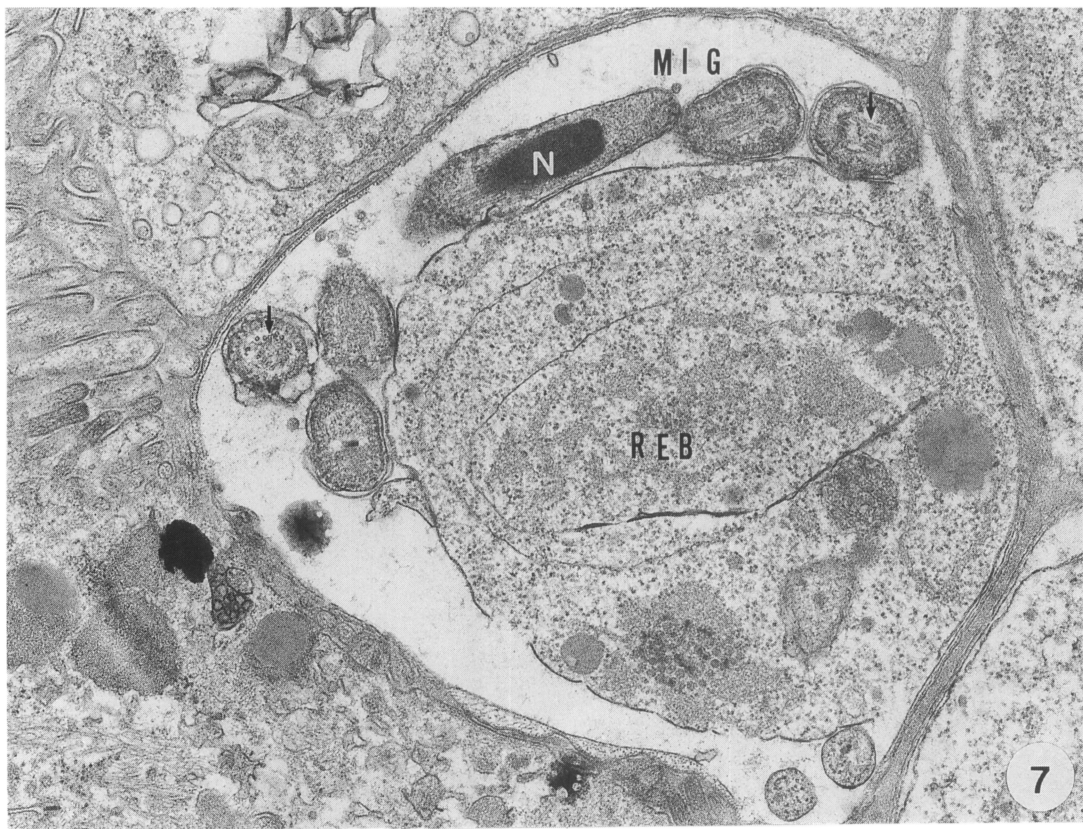
did not develop clinical signs of disease or growth retardation (Tzipori *et al.*, 1981). Studies of *C. parvum* infection in several strains of laboratory mice have demonstrated that 4- to 6-day-old mice were more susceptible to experimental infection than those of 21 or 42 days old (Sherwood *et al.*, 1982; Heine *et al.*, 1984). Sreter *et al.* (1995) indicated that both innate and acquired resistances to *C. baileyi* were age-related in chickens.

On the other hand, we corroborated that susceptibilities to experimental infection with *C. muris* (strain MCR) in mice at 3, 12 and 15 wk of age were similar (Rhee *et al.*, unpublished observations). *Cryptosporidium muris* appears to be associated with mild diarrhea in cattle of all ages, especially young animals (Upton and Current, 1985). Anderson (1988) described that abomasal cryptosporidiosis was produced in only one of 18 calves exposed 2 hr pre-clostrally to millions of fresh oocysts without presenting data in details. Given the data presented here, although it is well known that younger animals are more susceptible to *Cryptosporidium* infection, susceptibility to *C. muris* infection in kids and lambs may be irrespective of age like mice, because of a low relationship between age and intensity of oocyst outputs. However, we could not conclude age-independent resistance to *C. muris* in kids and lambs because the number of experimental animals are not sufficient.

Infection with *C. muris* appears to be chronic in cattle: the pattern of shedding oocysts in the steers was continuous over a period of 4 months and as of his writing, he had possession of three cattle which had shed *C. muris* oocyst for over a year at the rate of about one million per gram of feces (Anderson, 1987 & 1989). However, the prepatent and patent periods of the parasite for ruminants have not been established throughout transmission experiment as yet.

Our previous work (Rhee *et al.*, 1995) revealed that a patent period ranged from 61 to 64 days with a prepatent period of 9-11 days for mice inoculated orally with various doses of *C. muris* (strain MCR) oocyst. While, Iseki *et al.* (1989) intimated that the prepatent and patent periods of *C. muris* (strain RN 66) for mice, guinea pigs, rabbits, dogs and cats were exceedingly different from each other. Based on the present findings, the prepatent and patent periods for kids and lambs were longer than those reported for various laboratory animals by Iseki *et al.* (1989) and Rhee *et al.* (1995).

The significant differences in patency and the total oocyst outputs were noted depending on individual hosts. The standard deviations of the means of patency and the total oocyst outputs were high (47.8 ± 21.1 days and 156.6 × 10^6 ± 253.6 × 10^6), whereas the lengths of the prepatent period were similar to one another. Those of the prepatent period was relatively low (28.1 ± 5.8 days), while there was no correlation between the length of the patent period and the total oocyst output, indicating a significant individual difference in terms of the effectiveness of the acquired resistance to *C. muris* (Table 1). Therefore, even by same strain of inoculum, the prepatent and patent periods of *C. muris* vary significantly with the species of host.

In the present study, no oocysts were excreted from a lamb and a kid by challenge infections at any time, which implies that the

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Fig. 3-8. Transmission electron micrographs showing the endogenous development of *C. muris* (strain MCR) parasitizing the abomasum of a kid. **Fig. 3.** A trophozoite growing in the microvilli of the surface mucus cells of the abomasal peptic glands, × 10,000. **Fig. 4.** An indented border separating the parasite projection consisting of feeder organelle from the filamentous process of the host cells, × 12,000. **Fig. 5.** A younger meront growing in the microvilli, showing a banana-shaped and four round cross-sections of merozoites, × 6,000. **Fig. 6.** A macrogamete showing numerous amylopectin bodies in the microvilli, × 8,000. **Fig. 7.** A microgametocyte comprising eight microgametes in the microvilli, × 10,000. **Fig. 8.** An oocyst at the late stage in the microvilli, showing large parasitophorous vacuole, × 6,000.
Abbreviations: A, annular ring; AP, amylopectin body; D, dense band; F, filamentous process; FO, feeder organelle; IB, indented border; MC, mucus cell; ME, merozoite; MIG, microgamete; N, nucleus; NU, nucleolus; REB, residual body; PV, parasitophorous vacuole; PVM, parasitophorous vacuolar membrane; SP, sporozoite; VM, villous membrane; W, oocyst wall; WB, wall-forming body

immunogenicity of the parasite is very strong like mice. Moreover, we ascertained the infectivity of the oocysts discharged from the kids to mice and reappearance of latent cryptosporidia after primary infection by prednisolone acetate administration like mice. However, it is concluded that ruminants such as cattle, goat and sheep are not suitable hosts for the protozoon because infection with greater numbers of oocyst output was not found to occur with a familar findings in ruminants.

The present study primarily documented that the ultrastructural features of the attachment site of the parasite to the mucus cells differed remarkably from those of C. parvum in the abomasum of ruminants. A unique structure of the anterior projection of the parasite was seen identical with that observed in the stomach of mice, as previously described by Uni et al. (1987) and Rhee et al. (1991b). Moreover, the size of the parasite was greater at each developmental stage than that of C. parvum. In addition, massive populations of this cryptosporidia were seen in the abomasal peptic glands, most of which were found infected with them.

Therefore, the present paper clarified the full course of the host-parasite interaction in ruminants throughout the transmission experiment and ultrastructure of the developmental stages with confirming greater numbers of cryptosporidia in the gastric peptic glands and fecal samples of kids and lambs.

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=초록=

재래산양 및 면양에 있어서 쥐와포자충 (MCR주)의 감염 동태

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반추동물에 있어서 쥐와포자충의 감염 동태에 관한 지식에는 불충분한 점이 많다. 그래서 11마리의 1-20일령의 재래산양과 면양에 2×10^7의 쥐와포자충 (MCR주) 난포낭 (oocyst)을 한 번에 경구투여한 다음 분변 내의 난포낭 배설 양상을 관찰하였다. 11마리의 재래산양과 면양 중 4마리에서만 분변에서 억대 (10^8/day/head)에 이르는 난포낭이 검출되었는데 반해 3마리에서 천만대, 나머지 예에서는 극히 적은 수만이 검출되었다. 전기생충증명기는 19-35일로서 평균 28.1 ± 5.8일, 기생충증명기는 16-85일로서 평균 47.8 ± 21.1일, 분변 내 난포낭 배설량은 최고가 613.5×10^6, 평균 $156.6 \times 10^6 \pm 253.6 \times 10^6$이었으며, 이 원충에 대한 연령의존성 저항은 인정할 수 없었다. 한편, 감염 후 44일째 난포낭 배설 극기에 희생시킨 재래산양의 제4위 점액세포의 미세융모 내에서 이 원충의 모든 단계의 발육기를 관찰할 수 있었다. 모든 발육기에서 바깥쪽이 숙주세포의 두터운 사상돌기로 둘러싸여 있는 쥐와포자충 특유의 전단 돌출부를 볼 수 있었다. 또, 이 원충의 재래산양/마우스 모델 감염실험에서 마우스 고유의 난포낭 배설 양상이 인정되었다. 이 원충의 면역원성과 난포낭의 재생산이 도전감염실험과 면역억제에 의하여 증명되었다. 이상의 실험 결과로 반추수에 있어서 쥐와포자충의 내생발육을 입증하였다.